

Characterization of Eukaryotic Translation Initiation Factor 5A-2 (eIF5A-2) in *Arabidopsis thaliana*: Effects of Wounding and Pathogen Attack

by

Yulia Gatsukovich

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Abstract

Plants respond to wounding and pathogen ingress by synthesizing defence proteins that facilitate wound healing, cell death or provide protection against further pathogen attack. The accumulation of these proteins is mainly due to rapid transcriptional activation of corresponding genes, though preliminary evidence implies that translational control may also have a role in the stress response. This study focuses on the function of *Arabidopsis thaliana* eukaryotic translation initiation factor 5A (eIF5A) in mechanical wounding and during infection with the phytopathogen *Pseudomonas syringae*. Recent evidence suggests that eIF5A acts as a nucleo-cytoplasmic shuttling protein that facilitates mRNA translation through selective transport from the nucleus. Three isoforms of eIF5A have been identified and isolated in *Arabidopsis thaliana*, suggesting that each distinct isoform may be involved in transport of different subsets of mRNA required for a specific physiological event.

The analysis in this study revealed that the eIF5A-2 isoform appears to be involved in the signal transduction pathways that result in cell death following virulent infection, systemic acquired resistance and wounding in *Arabidopsis thaliana*. Transgenic plants with reduced expression of eIF5A-2 were developed using antisense T-DNA insertion. These lines have shown marked resistance to *Pseudomonas syringae* pv *tomato* DC3000, exhibiting up to 99% decrease of bacteria *in planta* and cell death associated with disease. The capacity to effectively limit growth of various pathogens is important for the design of strategies to improve disease resistance in crops. Development of disease-resistant lines allows efficient crop production with reduced reliance on environmentally undesirable toxic agrichemicals.

Dedication

I would like to dedicate this thesis to my Mom and to little brother Anton, who once told me that he would never be a “sheep”. It took me 5 years to realize what he meant by that. I have gained a new perspective on life thanks to him.

“...like a free spirit unfettered by positional possessions” - Orlando

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Introduction

The importance of autotrophic life forms, like plants, for the survival of the rest of life on the planet is well understood. Perhaps equally significant is our reliance on plants, not only for food, but also for building supplies, clothing, paper, furniture, and a multitude of other manufactured products. Related industries are negatively impacted by reduced plant growth caused by biotic stresses such as pathogen attack and herbivory, posing a major problem for society.

Most plants are naturally resistant to a plethora of pathogens found in the wild. By understanding this natural resistance and the sub-cellular interactions that occur when a plant system is attacked by a pathogen, one may be able to prevent disease by increasing the resistance of plants to pathogens. It may be possible to alter susceptible plants by genetically engineering them to express the genes that confer resistance.

1.1 Plant-Pathogen Interactions and the Gene-for-Gene Hypothesis

After entry of a pathogen, the plant is thought to respond to infection in one of three general ways. If the plant is not able to support the growth of a pathogen, it will not be able to replicate, thrive, and cause disease in the plant. This general response is known as passive non-host resistance (Heath, 2001).

On the opposite end of the spectrum, pathogens may be able to cause a systemic infection where the pathogen replicates and spreads from the initially infected cells to other cells of the plant. Plants infected in this manner show physical symptoms of disease. This disease may interfere with normal growth and reduce the plant's lifespan and/or productivity. The pathogen is allowed to multiply to high population levels within the apoplast, leading to a widespread chlorosis associated with the systemic spread of pathogen (Alfano and Collmer, 1997).

A third interaction that occurs between plants and pathogens is the ability of the plant to actively combat the pathogen through activation of specific disease resistance signaling. In this process of active resistance, the plant recognizes that it is being invaded by a foreign organism and immediately initiates a response by which the growth of the pathogen is suppressed. This is known as the gene-for-gene response. In this case, bacteria, viruses and fungi contain certain gene-products that host systems are able to recognize and, accordingly, react to by initiating defense responses. One manifestation of this is the hypersensitive response (HR) whereby at the point of entry, the cells immediately surrounding the infection site undergo programmed cell death causing necrotic lesions (Keen, 1990). The purpose of this cell death response in the event of a viral infection is to localize the invading pathogen in the dead tissue and prevent movement from cell to cell, which would result in systemic infection. In the case of larger pathogens like bacteria that cannot cross the cell wall/membrane and live in the extracellular space, HR prevents the pathogen from obtaining the nutrients needed to grow and multiply. HR seems to be induced by particular gene-products made by *Avr* genes in the pathogen, the first of which was isolated from *Pseudomonas syringae* pv. *glycinea* (Staskawicz et al., 1995). *Avr* gene-products are recognized *in planta* by plant resistance genes (*R* genes) that produce gene-products (*R* proteins). It has been proposed that there is either a direct or indirect interaction between the *R* gene products and *Avr* gene-products, which elicits the HR (Flor, 1971). The gene-for-gene model of HR is analogous to a receptor-ligand model; the *R* gene-product acts as receptor and the *Avr* gene-product acts as the ligand. However, if the interaction is not direct, a more complex model must be invoked to explain the interaction between *Avr* and *R* factors. The Guard Hypothesis proposed by Van der Biezen and Jones (1998) proposes that an elicitor protein produced by the pathogen binds to and inhibits the activity of a basal defense activator. The *R* gene-product then recognizes this complex, binds to it and activates the defense mechanisms.

1.1.1. Pathogen Avirulence Gene (*Avr* genes)

Avr genes produce elicitors that act either directly or indirectly as signal molecules interacting with R proteins in the host to elicit the defense response. In viral systems the elicitor is usually the direct product of the *Avr* gene whereas in bacterial systems the elicitor is usually a secondary messenger that is activated with the expression of the *Avr* gene. This stems from the idea that bacteria use different pathological mechanisms than their viral counterparts. Viruses attack cells from within the host cell whereas bacteria cannot typically penetrate the plant cell membrane and must remain in the extracellular space (apoplast). Therefore bacterial elicitor proteins must enter the cell through a completely different mechanism (Buchanan et al., 2000). This system is called the Type III effector secretion system where avirulence and virulence proteins are delivered to the host cell through hrp pili from the apoplastic space (He et al., 2001, Hueck, 1998, Galan, 1999).

To study gene-for-gene resistance, first, a single *avr* gene was isolated by introducing a cosmid library produced from an avirulent strain into a virulent strain. A cosmid clone containing an *avr* gene transformed the virulent strain to an avirulent strain. Use of such a strain carrying only a single *avr* gene created a situation in which only a single gene-for-gene interaction was operating in the plant-pathogen system. The avirulent strain of *P. syringae* pv. *tomato* DC3000 was developed in the laboratories of Fred Ausubel and Brian Staskawicz, who inserted the *avr* gene, *avrRpt2*, converting DC3000 into an avirulent strain in the *Arabidopsis* ecotype, Columbia (Dong et al., 1991, Whalen et al., 1991).

1.1.2. *RPS2/AvrRpt2* Gene-for-Gene HR Model

The gene in Col-0 required for recognition of the avirulence factor AvrRpt2 and elicitation of HR is *RPS2*, which encodes the RPS2 protein. There appears to be a direct interaction of RPS2 and AvrRpt2 inside the plant cell based on *in vitro* assays (Leister et al., 1996). It was thought based on

these same *in vitro* studies that RPS2 was located in the cytosol. However, recent direct *in vivo* evidence suggests that RPS2 is actually membrane-bound (Boyes, 1998, Axtell and Staskawicz, 2003).

Bacteria containing the plasmid vector expressing the avirulence gene, *avrRpt2*, elicit the normal gene-for-gene HR in *Arabidopsis* plants carrying the corresponding R gene, *RPS2* (Yu et al., 2000). The biochemical basis for HR and the corresponding resistance conferred by R proteins is just beginning to be understood. RIN4 is a factor that is physically associated with RPS2 *in vivo* and is required for proper RPS2 function (Mackey, 2003). RIN4 may act as a suppressor of RPS2, which is consistent with information showing that over-expression of RIN4 inhibits RPS2 function and elimination of RIN4 activates RPS2. In addition, AvrRpt2 induces, independently of RPS2, the disappearance of RIN4. Another factor, NDR1, is required for RPS2-mediated HR and resistance (Century, 1995).

1.1.3. Oxidative burst and other biochemical interactions of HR

One event associated with the hypersensitive response is the oxidative burst: the production of reactive oxygen species (ROS). These compounds include O_2^- , H_2O_2 , and perhaps $\cdot OH$ (Yahraus et al., 1995). Genetic evidence has been obtained that supports the idea that the oxidative burst is a downstream component of the *RPS2* (R gene)/*avrRpt2* (Avr gene) gene-for-gene signal cascade that leads to the HR (Wolfe et al., 2000). The oxidative burst occurs before the HR, and is not a byproduct of HR but rather seems to be a signaling factor for HR. It is possible that the oxidative burst is responsible for general disease resistance (Yahraus et al., 1995). There is also an association of salicylic acid (SA) accumulation with the defense responses to pathogens including the HR and systemic acquired resistance (SAR) (Delaney, 1994, Keen, 1990). In addition, nitric oxide (NO) also seems to play an important role in HR cell death (Delledone, 1998, Durner, 1998). Other events that

are associated with HR are increases in defense gene expression and strengthening of the cell walls with auto-fluorescent compounds such as lignin (Glazebrook et al., 1997).

1.1.4. Systemic Acquired Resistance

Plants in which HR has been induced by avirulent pathogens exhibit resistance to subsequent inoculations with virulent pathogens. Such resistance to later attack by pathogens is called systemic acquired resistance (SAR) (Kuc, 1995, Sticher et al., 1997). SAR has three stages: the induction/immunization stage that begins with reactive oxygen species (ROS) accumulation during HR, the establishment stage, which is accompanied by systemic micro-HR, and the manifestation stage, which occurs when the plant is challenged by a normally virulent pathogen (Alvarez, 1998). In *Arabidopsis* an accumulation of salicylic acid is required to induce SAR (Vernooij et al., 1994, Dong, 1998). One clear demonstration of this is the finding that repression of salicylic acid accumulation by expression of a bacterial salicylic acid hydroxylase gene (*NahG*) abolishes SAR (Delaney, 1994). SAR is also associated with the induction of several pathogenesis-related (PR) proteins (Lawton et al., 1995, Uknes et al., 1993). *PR-I* gene expression is considered a marker for general disease resistance and SAR.

1.2 Pathogen Virulence

To be a successful extracellular pathogen, virulent *P. syringae* pv. *tomato* DC3000 must evolve an array of pathogenic mechanisms to suppress or evade *Arabidopsis* defense responses that are apparently effective in preventing virulent infection by the vast majority of potential parasites. It must also develop mechanisms to release nutrients and water to the apoplast, where bacteria live. How *P. syringae* succeeds in doing this is not known. However, molecular genetic analysis of *P. syringae* pathogenicity has revealed two virulence systems that play an important role in *P. syringae* infection of plants: the type III protein secretion system and a toxin called coronatine.

1.2.1 The type III protein secretion system: A key pathogenicity factor in *P. syringae*

The type III protein secretion system was discovered first in the human pathogens *Yersinia* spp., but has now been found to be widespread among Gram-negative bacterial pathogens of plants and animals (He et al., 2001, Hueck, 1998, Galan, 1999). The most intriguing feature of this protein secretion system is its ability to actively inject bacterial virulence proteins directly into host cells. The importance of the type III secretion system in *P. syringae* pathogenicity is underscored by the observation that *hrp/hrc* mutations that block type III secretion completely eliminate *P. syringae* infectivity in susceptible *Arabidopsis* plants (Roine, 1997).

The ability of *P. syringae* to inject virulence proteins directly into the host cell is believed to be highly significant in pathogen evolution because this injection mechanism enables the pathogen to gain access to a vast number of intracellular host targets that would not be available for bacterial virulence proteins delivered to the surface of host cells. The majority of the known type III effectors are Avr proteins, which are identified based on their ability to trigger resistance responses following recognition by the *R* gene products in the incompatible interactions. How *P. syringae* delivers Avr and other type III effector proteins from its cytoplasm to the host cell cytoplasm is not known. However, a *P. syringae* surface pilus (called the Hrp pilus) assembled by the type III secretion system has been shown to play a key role in this process, possibly by providing a bridge for protein transfer (Roine, 1997, Wei, 2000, Hu, 2001, Jin et al., 2001).

How type III effectors function inside the host cell to promote plant susceptibility is still under investigation. To date, no plant ‘susceptibility’ pathway has been clearly identified in any plant-bacteria interaction. In animal-pathogen interactions, increasing evidence suggests that the MAP kinase defense pathway (Orth et al., 1999), delivery of reactive oxygen-generating enzymes (Vazquez-Torres et al., 2001, Vazquez-Torres and Fang, 2001), ubiquitin-like molecules (Orth et al., 1999), and actin cytoskeleton (Galan and Zhou, 2000) in the host are all targets of type III virulence

proteins, demonstrating that type III effectors are ‘smart bombs’ sent by pathogens. Unfortunately, the primary sequences of the identified *P. syringae* type III effector genes provide little clue to their functions in modulating plant signaling and metabolic processes. It is almost certain however, that a major function of *P. syringae* type III effector proteins is to suppress plant defense responses in the host. For example, *hrp* mutant bacteria appear to induce the expression of several general defense-associated genes, such as phenylalanine ammonia lyase and chitinase genes (Jakobek et al., 1993), as well as papillae formation (Bestwick et al., 1995). By contrast, the wild type strains suppress expression of these defense genes and the formation of papillae, the latter being structures composed of lignin and callose, often observed at the pathogen infection site between the primary cell wall and the plasma membrane of a host cell.

There is also bacterial genetic evidence that some *avr* gene products, including *AvrRpt2*, interfere with the function of other *avr* gene products in the elicitation of host resistance (Ritter and Dangl, 1996, Reuber and Ausubel, 1996, Chen et al., 2000, Tsiamis et al., 2000). As extracellular pathogens, *P. syringae* and other bacterial pathogens must also cause host cells to release water and nutrients into the apoplast. Consequently, some type III effectors may be involved in water and nutrient release. Despite these accumulating clues, in no case has a specific plant ‘susceptibility’ target been identified for any type III effector in plant pathogenic bacteria. Identification of host susceptibility targets/pathways is therefore a major challenge in the field.

1.2.2 The coronatine toxin-A molecular mimic of methyl jasmonate

In addition to the type III secretion system, strain DC3000 cells also produce a toxin (called coronatine) that plays a significant role in modulating host susceptibility (Bender et al., 1999). However, unlike mutations affecting the type III secretion system, mutations affecting coronatine production do not completely eliminate pathogen virulence in susceptible *Arabidopsis* plants. Rather they have only a partial effect on pathogen virulence. Most notably, coronatine-deficient bacterial

mutants cause substantially weaker disease symptoms (chlorosis and necrosis) and a subtle reduction of bacterial multiplication in host leaves (Bender et al., 1987, Mittal and Davis, 1995).

The structure of coronatine has two distinct components: polyketide coronafacic acid (CFA) and coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Katagiri et al., 2002). The primary symptom elicited by coronatine in plants is tissue chlorosis (Gnanamaickam et al., 1982). However, exogenously applied coronatine induces purpling of *Arabidopsis* leaves (Bent et al., 1992), presumably resulting from anthocyanin production. Coronatine also inhibits root elongation and stimulates ethylene production in plants (Ferguson and Mitchell, 1985)(Kenyon and Turner, 1992, Feys et al., 1994).

Coronatine bears remarkable structural and functional homologies to methyl jasmonate (MeJA). Furthermore, coronatine and MeJA induce similar biological responses in *Arabidopsis* seedlings (Feys et al., 1994) and other plants (Weiler et al., 1994, Greulich et al., 1995, Koda et al., 1996), leading to the suggestion that coronatine functions as a molecular mimic of MeJA. MeJA accumulates in response to wounding or insect chewing and is involved in a systemic defense response to invading insects via production of defense compounds, including proteinase inhibitors (Ryan and Pearce, 1998). A possible reason as to why bacteria would produce a toxin whose function mimics that of MeJA could be that coronatine somehow conditions host plants to be more susceptible to bacterial infection via activation of the JA signaling pathway. Emerging evidence suggests that JA-mediated insect defense and SA-controlled pathogen resistance are sometimes antagonistic to each other so that activation of one pathway leads to inhibition of the other (Felton et al., 1999a, Felton et al., 1999b, Pieterse and van Loon, 1999, Thomma et al., 2001). This has been interpreted as reflecting prioritization by plants of energy-consuming defense responses towards specific insults (e.g., insects vs. some microbial pathogens). If this is true, coronatine could act as a suppressor of the plant's SA-dependent microbial defense system by triggering JA-mediated insect defense response. Consistent

with this idea, it has been reported that a coronatine mutant of DC3000 induces the expression of two pathogen defense-associated genes (phenylalanine ammonia lyase and glutathione S-transferase) more strongly than wild type DC3000 (Mittal and Davis, 1995). However, the precise mechanism by which coronatine tricks *Arabidopsis* and other plants to turn on the JA pathway and to presumably inhibit effective plant defense against *P. syringae* is yet to be resolved.

1.3 Programmed Cell Death

Host cell death can occur in both susceptible (compatible) and resistant (incompatible) plant-pathogen interactions. Several studies indicate that cell death during the hypersensitive response involves activation of a plant-encoded pathway for cell death (Nishimura et al., 1987, Morel and Dangl, 1997, Heath, 1998). Many susceptible interactions also result in host cell death, although it is not clear how the host participates in this response. In either case, pathogen challenge can trigger groups of plant cells to die. The cellular characteristics of the death process strongly implicate specific signals and autonomous cellular biochemical processes that execute individual cells. Various findings show similarities in the microbial infection mechanisms of animals, humans and plants (Bergey et al., 1996, Whitham et al., 1994, Dwyer et al., 1996). An emerging question is whether common features also exist between programmed cell death pathways in plants and those in other eukaryotes. In animals, programmed cell death (PCD), or apoptosis, is genetically controlled cellular suicide essential for development, maintenance of cellular homeostasis, and defense against environmental insults including pathogen attack (Lockshin et al., 1998). Some features of PCD have been observed in both susceptible (Xu and Roossinck, 2000) and resistant (Ryerson and Heath, 1996) reactions during plant-pathogen interactions, suggesting that overlapping biochemical pathways are operative in these two contrasting outcomes. A recent study by Dickman et al. (2001) has demonstrated that tobacco plants expressing human Bcl-2 and Bcl-xl, nematode CED-9, or baculovirus Op-IAP transgenes, products of which are negative regulators of apoptosis, exhibited heritable resistance to

several necrotrophic fungal pathogens, suggesting that successful disease development required host–cell death pathways. This implies that in compatible (virulent) plant–pathogen interactions apoptosis-like programmed cell death occurs. Suppression of plant cell death may improve resistance to necrotrophic pathogens such as *P. syringae*.

1.5 *Arabidopsis thaliana* as a Plant Model for Pathogenesis Research

Arabidopsis thaliana is ideal for research on plant-pathogen interactions. The plant is compact in size, spanning only a few inches in diameter when mature, and has a relatively short life cycle of 5-6 weeks from germination to seed. It contains a genome of about 125 Mb in five chromosomes making it the smallest genome among known flowering plants. The total number of genes the plant contains is 25,498 (Sparrow et al., 1972, Leutwiler et al., 1984, Meinke et al., 1998). There are extensive amounts of natural variation in wild populations known as ecotypes making the plant suitable for molecular genetic studies. By comparing normal development of this plant to that of mutant strains, the normal functioning of its genome can be elucidated (Bowman, 1994).

The *Arabidopsis-Pseudomonas syringae* pathosystem is a widely recognized model for studying plant-pathogen interactions (Katagiri et al., 2002) since both the plant and the pathogen are amenable to rigorous genetic analysis. *P.syringae* pv. *tomato* DC3000 is one of the most commonly used strains of this necrotrophic pathogen. Upon entry into the host tissue through a wound or a stomatal opening, *P. syringae* multiplies to high population levels in the intercellular spaces, leading to formation of “water-soaked” patches on leaves within 46 h resulting from massive release of water and, presumably, nutrients from the host cells. The water-soaked patches develop into necrotic lesions surrounded by chlorosis at 72 h post infection (Katagiri et al., 2002).

1.5 Wounding

Wounding of plant tissue is a common result of abiotic stress factors such as wind, rain and hail, and also of biotic factors, especially insect feeding. A wide array of anatomic, metabolic and physiological changes occurs at the site of damage, where the wounded tissue undergoes cellular de-compartmentalization, release of stored materials and dehydration leading to localized necrosis (Leon et al., 1995).

Wounding presents a constant threat to plant survival because it not only physically destroys plant tissues, but also provides a pathway for pathogen invasion. To cope with wounding effectively, plants must prepare for pathogen attack while defending against insect predators. Therefore, it is hypothesized that plants have evolved mechanisms that integrate the pathogen and wounding responses. In support of this idea, studies have shown that wounding regulates a number of genes that are also regulated by, or play a role in, pathogen response (Reymond and Farmer, 1998, Durrant et al., 2000, Reymond et al., 2000). Wounding and pathogen responses also share a number of components in their signaling pathways (Maleck and Dietrich, 1999). For example, studies have shown that several plant hormones are important for wounding and pathogen responses. These include jasmonic acid (JA), salicylic acid (SA) and ethylene (Creelman and Mullet, 1997, Dong, 1998, Reymond and Farmer, 1998, Thomma et al., 1998). In particular, JA, a defined wounding hormone, is essential for certain pathogen responses (Dong, 1998, Thomma et al., 1998). Signaling pathways initiated by these hormones and pathogen infection have been further addressed by a recent study using cDNA arrays to identify genes commonly regulated by the hormones and an avirulent pathogen (Schenk et al., 2000). These analyses have indicated the existence of a substantial network of regulatory interactions and coordination during pathogen and wounding responses. Another study using a microarray approach focused on transcriptional profiling of genes after pathogen infection and identified regulons that are involved in systemic acquired resistance (Maleck et al., 2000).

Wounding may elicit pathways that interact with pathogen resistance and possibly other signaling pathways. Dissecting crosstalk between these pathways is critical to a full understanding of the plant response to environmental cues in general and to wounding in particular.

1.6 eIF5A

Eukaryotic translation initiation factor 5A, eIF5A, is one of the most highly conserved proteins between eukaryotes and archaeobacteria (Gordon et al., 1987, Bartig et al., 1992, Kyrpides and Woese, 1998). It carries a uniquely modified lysine that is referred to as hypusine [N- (4-amino-2-hydroxybutyl)-L-lysine], which is synthesized in a two-step reaction. First, the lysine residue is post-translationally modified through the addition of a butylamine group derived from spermidine by the enzyme deoxyhypusine synthase (DHS) forming a deoxyhypusine intermediate (Park and Wolff, 1988). This inactive intermediate form of eIF5A is then hydroxylated by deoxyhypusine hydrolase (DHH) resulting in the formation of the unique amino acid, hypusine (Park et al., 1993, Park et al., 1997). The hypusine residue forms an integral part of the activated eIF5A, which is the only known protein to contain it (Park et al., 1997). This protein is ubiquitous in eukaryotes but appears to be absent from bacteria. However, a similar facilitating function may be performed in bacteria by translation factor EF-P (Kyrpides and Woese, 1998).

Little is known about eIF5A in plants, although it has been cloned from alfalfa, tobacco, maize and tomato (Pay et al., 1991, Chamot and Kuhlemeier, 1992, Dresselhaus et al., 1999, Wang et al., 2001). The available data are restricted to sequence information and some tissue expression analyses. Transcript analysis revealed that eIF5A proteins are 80-97% identical across plant species and share 50-60% homology with eIF5A found in the animal kingdom, suggesting their importance in regulation of basic cellular processes. Moreover, the sequence of amino acids surrounding the hypusine residue is strictly conserved, signifying the importance of the hypusine modification throughout evolution.

Various sources have reported the existence of different isoforms of eIF5A. For instance, there are two known isoforms in humans, three in *Arabidopsis*, and four in lettuce (In et al., 1997, Wang et al., 2001, Wang et al., 2003). The isoforms from each species display a high degree of amino acid identity. The three isoforms of eIF-5A in *Arabidopsis* share 82-84% sequence identity at the nucleotide level and 82-87% identity at the amino acid level (Thompson et al., 2004).

Characterization of these isoforms will help identify their role in plant adaptation to stress and resistance to disease.

1.6.1 Proposed function of eIF5A in plant defense response

Originally, eIF5A was described as a translation initiation factor due to its ability to stimulate the synthesis of methionyl-puromycin *in vitro* (Benne and Hershey, 1978, Park et al., 1993). However, depletion of this factor in yeast caused only a small (30%) reduction in the protein synthesis rate (Kang and Hershey, 1994). These results have been used to argue against eIF5A as a translation initiation factor for general protein synthesis. More recent evidence suggests that eIF5A facilitates specific protein synthesis by promoting nuclear export of specific mRNAs (Bevec and Hauber, 1997). The high conservation of the N-terminus, containing the site of hypusination, and the variance in the amino acid sequence within the C-terminal end, where the putative RNA binding sites reside, suggest that the protein may have a regulatory function with specific RNA translocation capabilities (Thompson et al., 2004). Moreover, Bevec and Hauber (1997) demonstrated that eIF5A promotes protein translation through nuclear export of mRNA. Specifically, eIF5A has been identified as a cellular cofactor involved in nuclear export of the HIV type 1 Rev trans-activator protein. In human T cells, constitutively expressed mutants that were shown to have an inactivated chromosomal copy of eIF5A, but maintained binding capability to the HIV-1 Rev response element:Rev complex, displayed inhibited viral replication (Bevec et al., 1996). Therefore, human eIF5A may form part of a specific nuclear export pathway that is exploited by the virus for propagation. In addition, eIF5A has

been shown to interact with the nuclear export receptor CRM1 and is localized to both the cytoplasmic and nuclear compartments, with particular accumulation at the nucleoplasmic face of nuclear pore complexes (Rosorius et al., 1999). These findings, together with the discovery of various isoforms of eIF5A, have given rise to the distinct possibility that each isoform is responsible for transporting a unique subset of mRNA species required for a specific physiological event, such as cell division or cell death (Park et al., 1993, Kang and Hershey, 1994, Park et al., 1997, Wang et al., 2001). In effect, each eIF5A isoform is thought to act as a shuttle protein that initiates the translation of a developmentally required suite of genes.

Little is known about the elements that regulate the expression of eIF5A in the cell. However, it is clear that the ratios of the isoforms change when a cell enters a new stage of development and a specific suite of genes is expressed. For example, Wang et al (2001) observed that the transcript levels of eIF5A1 isoform increased during natural and stress-induced senescence in tomato (Wang et al., 2001). A similar trend was demonstrated in dinoflagellates where transcription of eIF5A was up-regulated at early G(1) and decreased dramatically on the entry to S phase (Chan, 2002).

In the present study, a specific isoform of eIF5A involved in signaling during pathogen challenge and wounding has been identified in *Arabidopsis*. The protein was shown to be strongly up-regulated during virulent infection with *Pseudomonas syringae* pv. *tomato* DC 3000 as well as in response to wounding. Transgenic plants with suppressed eIF5A gene expression exhibited abrogation of disease development in response to virulent infection, demonstrated by reduced bacterial colonization and subsequent lack of chlorosis. Since, *P. syringae* is a necrotroph, which requires initiation of plant cell death to colonize in the plant, its inability to multiply in transgenic plants with suppressed eIF5A protein suggests that the protein may play an important role in cell death induced by virulent infection.

Materials and Methods

2.1 Plant Material

Seeds of *Arabidopsis thaliana* ecotype Columbia were sown in soil (Premier Pro-Mix BX, Premier Horticulture) and maintained in a growth chamber at 20 °C under photoperiod cycles of 16 h light and 8 h darkness at 75% RH for wounding and infection experiments. In some experiments, plants grown under short-day conditions were used for infection. In this case, the plants were grown under photoperiod cycles of 9 h light and 15 h darkness at 75-80% RH. All plants were treated at the age of 4 weeks.

2.2 Isolation of Genomic DNA

Genomic DNA was isolated using a Promega Wizard® Genomic DNA Purification Kit according to manufacturer's instructions, with modifications. Fresh rosette leaves, harvested from wild type 4-week old *Arabidopsis thaliana* plants were finely ground with a mortar and pestle in excess liquid nitrogen and transferred to a 1.5ml microcentrifuge tube containing 600 µl of homogenization buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 250 mM NaCl, 1% (v/v) 2-β-mercaptoethanol). This suspension was incubated at 65° C for 15 minutes and treated with 3 µl of RNase Solution (4 mg/ml) for 15 minutes in a 37° C water bath. After subsequent cooling to room temperature for 5 minutes, 200 µl of Protein Precipitation solution was added to the suspension and vortexed on high speed for 20 sec. The sample was then centrifuged at 13,000 rpm for 3 minutes to precipitate the protein. The supernatant containing the DNA was collected and transferred to a new 1.5 ml microcentrifuge tube containing 600 µl of room-temperature isopropanol. The tubes were inverted several times until the DNA became visible as thread-like strands and then centrifuged for 1 minute at 13,000 rpm. The supernatant was discarded and the DNA pellet was resuspended in dH₂O.

2.2.1 PCR of 3'UTR eIF5A-2 cDNA

Using genomic DNA as the template, the eIF5A-2 3'-untranslated region (UTR) was isolated and amplified by PCR. Upstream and downstream primers were designed by Marianne Hopkins (Dr. J.E. Thompson's Lab) based on the sequences available in GenBank (accession # BE039424). The primers used for the amplification contained the appropriate endonuclease restriction sites (*SacI* and *XhoI*) for subsequent cloning into the binary vector pKYLX71-35S² :

5'-GAGCTCGTTGGTGGTGGCAAGTAAACAAGTATC-3'

5'-CTCGAGGGAAGTCTTGCAACAAGAAACACAC-3'

The primers were synthesized by Sigma Genosys (Oakville, Ontario). PCR of the 3' UTR of eIF5A-2 was performed with a programmable Perkin Elmer GeneAmp PCR 2400 thermal cycler. The PCR fragments were separated by electrophoresis in a 0.9% (w/v) agarose gel and examined with an Alpha Innotech Fluorchem 8000 chemiluminescence and visible imaging system for visual identification of resultant bands in comparison with molecular weight marker X (Roche Diagnostics, Canada).

2.3 Treatment with *Pseudomonas syringae*

2.3.1 Bacterial Growth Conditions

Two bacterial strains of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) were used to induce a pathogenesis response in *Arabidopsis* plants: virulent *Pst* DC3000 (pVSP61) and avirulent *Pst* DC3000 (pVSP61 + *avrRpt2*) (both obtained from Dr. Robin Cameron, University of Toronto, Toronto, Canada). Bacterial cultures were grown using King's B media (Whalen et al., 1991). Media was prepared by dissolving all components except antibiotics, and autoclaving for 30 to 60 min. Sterile kanamycin stock solution (50 mg/ml) was added to the autoclaved media at a final concentration of 50 µg/ml using aseptic technique. Liquid media was dispensed in 5 ml aliquots to

culture tubes and stored at 4°C. King's B media was inoculated with -80°C glycerol stock of *Pst* and grown with shaking at 28°C for 16 h until the cultures reached mid-log growth phase ($OD_{600} = 0.134$), which ensures the viability of bacteria and thus more consistent symptoms in *Arabidopsis* once it is inoculated. The cultures were then centrifuged at 4000 rpm for 7 min, and the bacterial pellets were each resuspended in 5 ml of 10mM $MgCl_2$. The inoculum was made by calculating the proper dilution necessary for 10^6 cfu/ml bacterial concentration.

2.3.2 Pathogen Ingress

Rosette leaves of 4- week-old plants were treated using one of four treatments: no inoculation, mock-inoculation with 10mM $MgCl_2$, inoculation with avirulent *Pst* strain (10^6 cfu/ml 10mM $MgCl_2$), or inoculation with virulent *Pst* strain (10^6 cfu/ml 10mM $MgCl_2$). The leaves selected for inoculation were marked using a permanent marker so that they could be identified later. The bacterial suspensions were then pressure-infiltrated into the abaxial surface of four rosette leaves of each plant using a 1 ml syringe without a needle. Care was taken to avoid injection into the vascular system. Approximately 10 μ l of inoculum can be infiltrated before the leaf is fully soaked. The intercellular spaces of the infiltrated leaves were allowed to dry and then the plants were placed back in the growth chamber for 3 days. The inoculated leaves were harvested at 0h, 24h and 72h for subsequent analysis.

2.3.3 *In planta* Bacterial Growth Assays

To isolate and quantify bacteria from the infected plants, leaves were surface-sterilized in 50% ethanol, followed by two rinses with sterile water. Six 0.4 cm diameter leaf disks per replicate were excised using a cork borer and placed in an Eppendorf tube. The leaf discs were homogenized in 500 μ L 10 mM $MgCl_2$ using a drill fitted with a blue plastic bit and then diluted 10^{-2} and 10^{-4} in 10mL of 10 mM $MgCl_2$ solution. A 0.1 mL sample of each dilution was distributed evenly on a King's B rifampicin/kanamycin plate. Each assay contained three replicates per treatment. (Preparation of 1L

King's B rifampicin/kanamycin medium: 20 g protease peptone, 10 mL glycerol, 1.5 g K₂HPO₄, 15 g bacteriological agar and 1 L dH₂O were autoclaved for 20 min and allowed to cool. 6 mL MgSO₄, 1 mL kanamycin (50 mg/mL) and 4 mL rifampicin (25 mg/mL) were added before the plates were poured). The plates were kept in the dark at 25°C, and colony-forming units (cfu) were determined for each plate.

2.4 Wounding

Four-week-old plants were wounded by crushing with a hemostat along the midvein (approximately 20 % of leaf surface) according to Stotz et al., (1999). Tissue was harvested at 10 min, 30 min, 1h, 2h, 3h, and 5h after wounding, immediately frozen in liquid N₂ and stored at -70°C for future analysis.

2.5 RNA Protocols

2.5.1 RNA Extraction from Plant Tissue

Total RNA was extracted from leaves of *Arabidopsis thaliana* transgenic and wild type plants as described by Chirgwin *et al.* (1979) and modified by Ohan and Heikkila (1993): The rosette leaves of frozen wild type and transgenic *Arabidopsis thaliana* plants were ground into a fine powder in liquid nitrogen using a mortar and pestle. 10 mL of guanidine isothiocyanate buffer was added (4 M guanidine isothionite (GIT) enzyme grade, BRL# 5535 U, 25 mM sodium acetate pH 6, 120 mM β-mercaptoethanol, in 0.1% (v/v) diethyl pyrocarbonate (DEPC)-treated water), and the samples were vortexed. The samples were then centrifuged at 7700g for 10 min using a Sorvall SS34 rotor (Mandel Scientific Co. Ltd., Guelph, ON). Afterwards, the supernatant was carefully layered onto 3.3 mL of filter-sterilized cesium chloride buffer (5.7 M CsCl optical grade, BRL# 55074A, 25 mM sodium acetate pH 6, in 0.1 % (v/v) DEPC-treated water) in Ultra-clear® ultracentrifuge tubes (14x89 mm, Beckmann, Palo Alto, CA). To pellet the RNA, samples were centrifuged at 111 000g for 23h at 20°C

in a Beckmann L8-70 ultracentrifuge using a SW-41 Ti rotor (Beckman, Palo Alto, CA). The buffer was gently removed, and the tube was inverted to drain any residual buffer. The resulting RNA pellet was rinsed with ice cold 70% (v/v) ethanol and inverted to drain for 10 min. The pellet was then resuspended in 360 μ L TES buffer (10 mM Tris HCl pH 7.4, 5 mM EDTA, 1% (w/v) SDS [sodium dodecyl sulfate]), transferred to a 1.5 mL Eppendorf tube and ethanol-precipitated by adding 100% ice-cold ethanol. The RNA was allowed to precipitate overnight at -20°C. The next day the RNA was isolated by centrifugation at 14,000 rpm for 10 min. The resulting pellet was resuspended in 20 μ L DEPC treated water.

2.5.2 Northern Blot Analysis

2.5.2.1 RNA Gel Electrophoresis

The resuspended RNA was fractionated by denaturing 1% formaldehyde-agarose gel (1% agarose, 5 mL 10x MOPS [0.2 M MOPS, 50 mM sodium acetate·3H₂O, pH 7, 10 mM EDTA], 36 mL DEPC treated water) electrophoresis. 10 μ g of RNA per sample was mixed with 20 μ L denaturing solution (50% (v/v) formamine, 16% (v/v) formaldehyde, 10% (v/v) 10x MOPS, 6.7% (v/v) DEPC-treated water, 5% (v/v) bromophenol blue [1%]). Ethidium bromide (0.01%) was included (0.5 μ L per sample) to verify equal loading of RNA, and electrophoresis was performed at 75 volts for 2 h (running buffer: 1x MOPS). The fractionated RNA was then transferred onto a Hybond™-N nylon membrane (Amersham, Pharmacia Biotech Inc., Piscataway, NJ). After transfer, the RNA was cross-linked to the damp membrane using a Bio-Rad Gene Linker UV Chamber set at C3 cross-linking setting. The membrane was pre-hybridized at 42°C for two hours with 6X SSC, 2X Denhart's Reagent, 0.1% SDS, and 100 μ g/ml denatured fragmented salmon sperm DNA before adding the ³²P-labeled probe.

2.5.2.2 Preparation of Radiolabelled DNA probe

The RNA blot was probed with ^{32}P -labeled *Arabidopsis* 3'UTR cDNA of eIF5A-1, 2, or 3. The ^{32}P was incorporated into the DNA using a random- primer DNA labeling kit according to the manufacturer's instructions (Boehringer-Mannheim/Roche). In detail, 250ng of DNA in a total volume of 10.5 μl of dH_2O was heated for 10 min at 100°C and rapidly cooled on ice in order to denature the DNA. The nucleotides dTTP, dGTP, dCTP as well as random hexamers were combined with the DNA. Behind a plexi-glass shield in a designated hood, [$\alpha^{32}\text{P}$] dATP (~50 μCi) and Klenow fragment DNA polymerase (Roche) were carefully added to the reaction, which was allowed to incubate for 1h at 37°C. TE buffer (10mM Tris, 1mM EDTA pH 8.0) was added to stop the reaction, and the unincorporated dNTPs were removed by passing the mixture through a Sephadex G50 column equilibrated with TE buffer. To determine the concentration of the radiolabelled probe, the amount of ^{32}P was measured using a liquid scintillation counter (Beckman model LS7000). A volume of labeled probe corresponding to 1×10^6 CPM was then used to probe the RNA blot.

2.5.2.3 Hybridization

The labeled probe was denatured by boiling and cooling on ice and added to the pre-hybridization solution. The hybridization was allowed to proceed overnight while rotating in the hybridization oven set at 42°C. After incubation, the membrane was washed for 20 min at 60°C with 2x SSC and 0.1% SDS, and then for a further 15 min at 60°C with 0.5x SSC and 0.1% SDS. The washed membrane was exposed to film (Kodak XL-1 Blue) at -80°C for 2 days with intensifying screens. The film was developed according to standard protocol.

2.6 Protein Protocols

2.6.1 Polyclonal Antibody Production

The aligned amino acid sequences of eIF5A from *Arabidopsis thaliana* are presented in Figure 1. The alignment was performed using MultAlin version 5.3.3 (Corpet, 1988). The highlighted regions represent the identical amino acid residues within the sequences of the three isoforms of eIF5A. The isoform-specific peptide sequences were designed by Marianne Hopkins (Dr. Thompson's lab) and contained the following amino acid sequences: eIF5A-1 – CNNDTLLQQI; eIF5A-2 – CTDDGLTAQM; eIF5A-3 – CTDEALLTQL. Note that the sequences chosen show the lowest degree of sequence identity.

All amino acid sequences were deduced from the nucleotide sequences of isolated eIF5A genes, and were obtained from NCBI databanks (www.ncbi.nlm.nih.gov). The peptide for eIF5A-2 was synthesized in the laboratory of Dr. G. Lajoie in the Department of Biochemistry at the University of Western Ontario. This 11-amino acid peptide was selected on the basis of the following criteria: hydrophilicity, which indicates that the peptide produced will be antigenic, and uniqueness. Finally, the cysteine residue located at the C-terminus of the peptide was used for conjugating the peptide through the sulphydryl group of the cysteine, to a carrier protein Keyhole Limpet Hemocyanin (KLH) prior to being used as an antigen for polyclonal antibody production, using the glutaraldehyde conjugation method of Drenckhahn et al. (1993). KLH was dissolved in phosphate-buffered saline (PBS; containing [per liter] 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄ and 2.4 g KH₂PO₄, pH 7.4) to a concentration of 1 mg/mL, and an equal amount of peptide was then added to the mixture. An equal volume of 2% (v/v) glutaraldehyde (EM grade) was then added drop-wise, with constant stirring, and the solution was mixed gently for 1 h at 4°C. The reaction was stopped by the addition of sodium borohydride to a final concentration of 10 mg/mL and then incubated for 1 h at 4°C with gentle mixing. The conjugated peptide solution was then dialyzed overnight against PBS at 4°C.

Antibodies were raised in rabbits using Freund's complete adjuvant for the first immunization and Freund's incomplete adjuvant for subsequent booster injections, according to the University of Waterloo Animal Care Standard Operating Procedures, in the animal care facility in the Department of Biology (AUPP# 9561).

2.6.2 Antibody Purification

Purification of polyclonal antibodies was performed using Sulfo Link Coupling Gels (Pierce) according to the manufacturer's instructions to eliminate cross reactivity to other non-specific proteins (e.g. Rubisco), thus increasing the sensitivity of Western blot analysis. The synthesized peptide used for antibody production was coupled to an affinity SulfoLink Coupling Gel column (Pierce) via the cysteine residue on the amino- terminus of the peptide. The SulfoLink Coupling Gel binds specifically to sulfhydryl groups under alkaline conditions and immobilizes any molecule with a free sulfhydryl (Pierce). Serum containing polyclonal antibodies was then applied to the column and incubated for 1h to allow the antigen and eIF5A-2 antibody to interact. The unbound material was washed out of the column using PBS buffer. The antibody was then eluted using low pH glycine buffer.

2.6.3 Protein Isolation

To extract the protein, leaf tissue was ground up in liquid nitrogen and suspended in homogenizing buffer containing 250 mM Sorbitol, 50 mM EPPS, 10 mM EDTA, 2 mM EGTA, 1mM DTT, 10 mM amino-n-carpoic acid [pH7.4]. The homogenate was centrifuged for 1 min at 13 000g to pellet the debris and the supernatant was decanted into a new Eppendorf tube.

Figure 1. Alignment of eIF5A proteins from *Arabidopsis thaliana*.

The highlighted regions represent the identical amino acid residues in the three isoforms of eIF5A.

The 11-amino acid synthetic peptide that was used to generate eIF5A-2-specific polyclonal antibody (TDDGLTAQMRIC) is delimited by the red box. Note that the sequences chosen show the lowest degree of sequence identity. All amino acid sequences were deduced from the nucleotide sequences of isolated eIF5A genes, and were obtained from NCBI databanks (www.ncbi.nlm.nih.gov).

1 MSDEHHHFESSDAGASKTYPQQAGTIRKNGYIVIKNRPCKVVEVSTSKTGKHGHAKCHFV
 2 MSDDEHHFEASESGASKTYPQSAGNIRKGGHIVIKNRPCKVVEVSTSKTGKHGHAKCHFV
 3 MSDDEHHFESSDAGASKTYPQQAGNIRKGGHIVIKGRPCKVVEVSTSKTGKHGHAKCHFV

1 AIDIFTSKKLEDIVPSSHNCVPHVNRDQYQLIDISEDGYVSLTDTNGSTKDDLKLP **NDD**
 2 AIDIFTAKKLEDIVPSSHNCVPHVNRVDYQLIDITEDGFVSLTDTSGGTKDDLKLP **TDD**
 3 AIDIFTSKKLEDIVPSSHNCVPHVNRVDYQLIDISEDGFVSLTDTNGSTKDDLKLP **TDE**

1 **TL**LOOIKSGFDDGKDLVSVMSAMGEEQINALKDIGPK
 2 **GLTAQMRI**GFDEGKDIVVSVMSMGEEQICAVKEVGGGK
 3 **ALLTQLKN**GFEEGKDIVVSVMSAMGEEQMCALKEVGPK

2.6.3.1 Protein Quantification

Protein concentration was determined according to (Ghosh et al., 1988) using bovine serum albumin as a standard. Samples were diluted in an equal volume of SDS-PAGE sample buffer (125 mM Tris-HCl (pH 6.8), 0.2% (v/v) glycerol, 4% (w/v) SDS, 0.1% (v/v) 2- β -mercaptoethanol and 0.0025% (w/v) bromophenol blue) and 2 μ L dots of the mixture were placed on Whatman 3M blotting paper. After allowing time for the spots to dry, the blotting paper was incubated in stain (40% (v/v) methanol, 10% (v/v) acetic acid, 0.2% (w/v) Coomassie brilliant blue R-250) for 20 minutes at room temperature, with gentle mixing. The paper was then incubated in several changes of destain solution (40% (v/v) methanol, 10% (v/v) acetic acid) to remove background stain from the blotting paper. Densitometry and comparison to a series of BSA standards enabled determination of protein concentration. Specifically, an image of the blot was captured using an HP ScanJet 3p scanner connected to a computer, and the integrated volume of each spot was measured using ImageQuant computer software.

2.6.4 SDS-PAGE and Western Blot Analysis

Polypeptides were fractionated by SDS-PAGE in Mini Protean Dual Slab Cells (Bio-Rad) using 0.75 mm thick 4% acrylamide stacking gels and 12% acrylamide separating gels (Laemmli, 1970). Following separation, proteins were transferred to nitrocellulose paper (0.45 μ m pore size, Bio-Rad) in transfer buffer comprised of 25 mM Tris, pH 8.3, 160 mM glycine, 0.002% (w/v) SDS and 20% (v/v) methanol. The transfer was conducted in a Bio-Rad Mini Trans-Blot transfer cell for 30 min at 20V (using a Bio-Rad model 250/2.5 power supply). Prestained molecular weight standards (Bio-Rad) were used to confirm successful transfer. Equal protein loading was verified by staining a second identical SDS-PAGE gel with Coomassie Brilliant Blue R250.

Following transfer, the protein blots were incubated for 1 h with gentle shaking in phosphate-buffered saline (10mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, 136mM NaCl, and 1% tween-20) containing 5% milk. Primary antibody was then added, and the blots were incubated for 1h at room temperature with gentle shaking. Following three 10 min washes with PBS-T, the blots were treated with goat anti-rabbit IgG secondary antibody and conjugated to alkaline phosphatase diluted 1:2500 in PBS-T for 1 h. Following this incubation, the blots were washed 4 times in PBS-T for 10 min. Secondary antibody that had bound to primary antibody was then visualized by incubating the blot in 10 mL of alkaline phosphatase buffer (100 mM Tris (pH 9.5), 100 mM NaCl, 5 mM MgCl₂) containing 100 µL nitroblue tetrazolium (50 mg/mL in 70% (v/v) dimethyl formamide) and 100µL 3-bromo-4-chloro-indolyl phosphate (50 mg/mL in dimethyl formamide) until bands could be seen. The reaction was stopped by rinsing the blots in distilled water.

2.6.5 Immunolocalization

Arabidopsis leaves infiltrated with *Pseudomonas syringae* were detached from the rosettes, and one 4 mm disk was excised from each leaf using a cork borer. The leaf disks were fixed in 4% paraformaldehyde and 1xPBS (pH 7.0) for 1h under vacuum, then placed in fresh fixative solution and incubated overnight at 4°C. After washing in 1xPBS (3 times for 30 min each), the samples were gradually dehydrated to 100% EtOH (20 min for each step) and rehydrated back to 100% H₂O. The disks were then placed into wells of a 6-well plate and blocked for 1h in PBS-T plus 5% BSA. After blocking, the samples were incubated overnight with eIF-5A2 antibody at 1:100 in PBS-T plus 1% BSA at RT, washed in PBS-T and incubated with anti-rabbit FITC conjugate antibody (Sigma) (1:100) for 1h in the dark. After washing in PBS-T, the samples were observed using a Zeiss LSM 510 confocal laser scanning microscope attached to an axiovert-inverted microscope.

2.7 Chlorophyll Analysis

Chlorophyll was extracted and quantified as described by Porra et al. (1989) with modifications. Individual inoculated leaves were harvested, weighed and then placed into DMSO and incubated at 60°C for 30 min. Absorbance of the samples was measured at 647 nm and 664 nm using a Beckman DU-64 spectrophotometer. Total chlorophyll concentration was calculated using the following formula:

$$\text{Chlorophyll a+b } (\mu\text{g/ml}) = [(17.67 \times A_{647}) + (7.12 \times A_{664})] \times \text{Dilution Factor}$$

2.8 Antisense 3'UTR eIF5A-2 Construct and Plant Transformation

A 141bp fragment containing the 3'UTR of eIF5A-2 cDNA (GenBank accession # BE039424) was amplified by PCR and subcloned into the *EcoRI* site of pGEM (Stratagene, La Jolla, CA). The resulting plasmid was digested with *XhoI* and *SacI* (Boehringer, Laval, QC), and the cDNA fragment was cloned into the *XhoI/SacI* site of the binary vector pKYLX71-35S² (Scharl et al., 1987). The pKYLX-asF5A-2 construct, which contains cDNA in the antisense orientation under the control of the cauliflower mosaic virus double 35S promoter, was introduced into *Agrobacterium tumefaciens* GV3101 by electroporation and transferred to *Arabidopsis thaliana* leaves by vacuum infiltration (Bechtold et al., 1993). The primary transformants (T₀) were allowed to mature and set seed in the growth chamber. T₁ seedlings were selected on Murashige and Skoog medium containing 50 mg/L kanamycin, 0.8% agar and 1% sucrose. The kanamycin-resistant seedlings were planted in soil and grown to maturity. T₂ and T₃ seedlings were similarly selected. Seedlings from T₃ lines that were 100% kanamycin-resistant were considered to be homozygous and were used for subsequent analysis.

2.9 Screening of T₃ Transgenic Lines.

The effects of infection with the bacterial pathogen *P. syringae* pv. *tomato* were compared for transgenic and wild type *Arabidopsis thaliana*. Seeds of transgenic and wild type plants were agitated

in 1 mL of seed sterilizing solution (15 mL bleach (6%), 0.05 mL Tween 20, and 40 mL sterile water) for 15 min. After discarding the sterilizing solution, the seeds were rinsed 5 times with sterile water, seeded on to MS plates (4.3 g Murashige & Skoog salt mixture, 20 g sucrose, 8 g phytagar, 1 mL 1000 x MS vitamins, 1 L sterile water and 100 mg kanamycin), and grown in a growth chamber at 22°C under 150 $\mu\text{mol}/(\text{m}^2\text{s})$ photosynthetically active radiation with 14h light and 10h dark cycles. After seven days, the plants were transplanted to soil (Premier Pro-Mix) and allowed to continue growing in the same chamber.

Infection of transgenic and wild type *A. thaliana* was carried out as described in section 2.3.

Results

The initial objective of this thesis was to analyze the expression patterns of eIF5A protein in *Arabidopsis thaliana* following pathogen infection and wounding. Since there is more than one isoform of eIF5A in *Arabidopsis*, the expression patterns of all three isoforms, AteIF5A-1, AteIF5A-2 and AteIF5A-3, had to be examined. Once the identity of the isoform involved in pathogen ingress and wounding had been established, a new objective arose: to determine the specific role of this eIF5A isoform in the signal transduction pathways leading to cell death following virulent infection, the hypersensitive response (HR) and wounding. To investigate this, transgenic plants exhibiting reduced levels of the pathogen/wounding eIF5A were constructed and tested for enhanced resistance to bacterial infection.

3.1 Identification of the eIF5A isoform involved in plant-pathogen interaction and mechanical wounding

3.1.1 Expression Analysis of eIF5A in *Arabidopsis* Challenged with *Pst* DC3000

Most interactions between plants and pathogens can be classified as either compatible or incompatible. During an incompatible interaction, the plant recognizes the pathogen and rapidly activates an extensive array of defense responses at the site of infection that limit pathogen ingress into neighboring cells. In contrast, a compatible interaction is characterized by a much delayed and attenuated defense response that fails to retard pathogen colonization. Based on the finding by Wang et al (2001), that a specific *Arabidopsis* isoform of eIF5A protein was up-regulated during natural senescence, a hypothesis was formed that the translation of proteins that participate in the pathogenesis responses may be facilitated by yet another isoform of eIF5A. The function of this

isoform of eIF5A would be to shuttle mRNAs transcribed in response to pathogen attack out of the nucleus into the cytoplasm for rapid translation.

To identify the isoform of eIF5A involved in plant-pathogen interaction, wild type *Arabidopsis* plants were inoculated with Pst DC3000, a model pathogen, at a density of 1×10^6 cfu/mL to allow for maximum bacterial multiplication within the host tissue. The expression of all three isoforms of eIF5A was then examined through northern and western blot analysis. The Northern blot analysis revealed that only one isoform, eIF5A-2, is expressed in *Arabidopsis* leaves (Fig 2). A single transcript of eIF5A-2, 0.7 kb in size, was readily detected in leaf tissue treated with either bacteria or $MgCl_2$. However, the eIF5A-2 transcript was also detectable in the leaves of untreated control plants indicating that it is constitutively expressed. There was some increase in transcript abundance for both mock- and avirulent-infected leaves, which may reflect a mild wounding response (Fig. 2).

That eIF5A-2 is involved in pathogen ingress was discerned from the Western blot analysis shown in Figure 3. Expression of eIF5A-2 at the protein level proved to be more selective than at the mRNA level with no significant up-regulation of the 18kD protein in mock infected leaves but clearly perceptible up-regulation at 24 hours post-inoculation with avirulent and virulent *Pst* (Fig. 3). Interestingly, the response to virulent bacteria resulted in still higher levels of eIF5A-2 protein at 72 hours post-inoculation (Fig. 3), which coincided with the appearance of disease symptoms in the form of necrotic lesions surrounded by diffuse chlorosis (Fig. 5). By 72 h post-inoculation, the expression of eIF5A-2 protein in leaves treated with avirulent bacteria had receded to background levels (Fig. 3). The single peak in expression observed around 24 hours following an infection with avirulent bacteria correlated with the formation of microscopic necrotic lesions associated with HR which were also evident at 72 h post-inoculation (Fig.5). Thus, the timing in up-regulation of eIF5A-2 following treatment with both avirulent and virulent bacteria coincides with host cell death.

Figure 2. Northern blot analysis comparing the expression of eIF5A isoforms in response to pathogen ingress in wild type *Arabidopsis*.

Total RNA was isolated from leaves of 4-week-old wild type plants inoculated with either 10mM MgCl₂ solution (Mock) or the avirulent strain of *Pst* DC3000 (Avr). The tissue was harvested immediately after the inoculation, 30 minutes after, and 34h post inoculation. The RNA was fractionated on a 1 % (w/v) agarose-formaldehyde gel, and probed with a 3'UTR of eIF5A-1, 2, and 3. The ethidium bromide stain of rRNA is shown for each lane as a loading control.

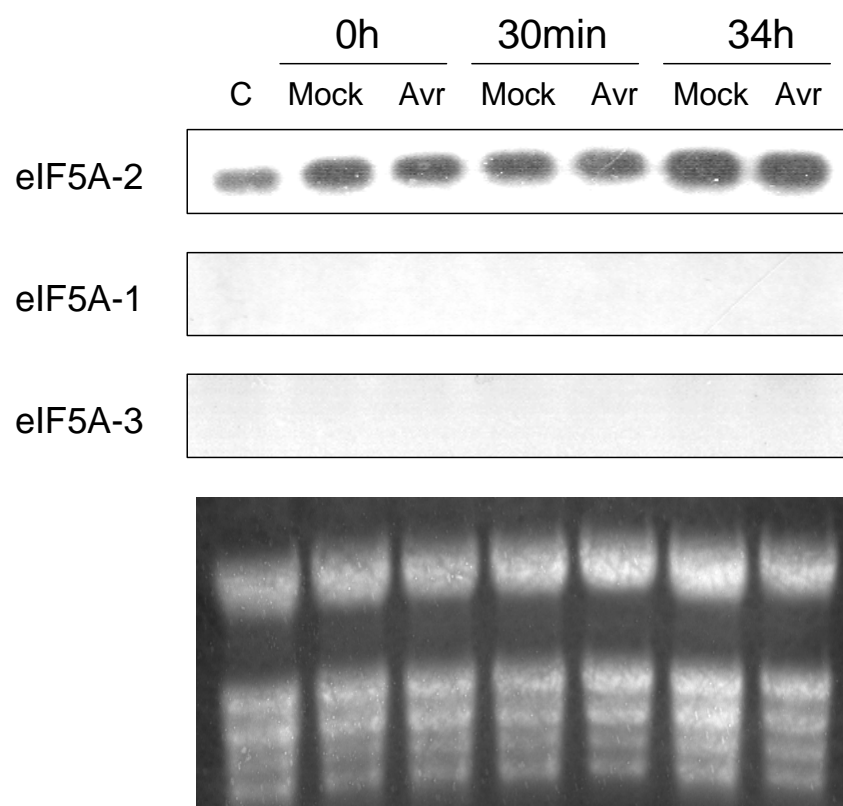
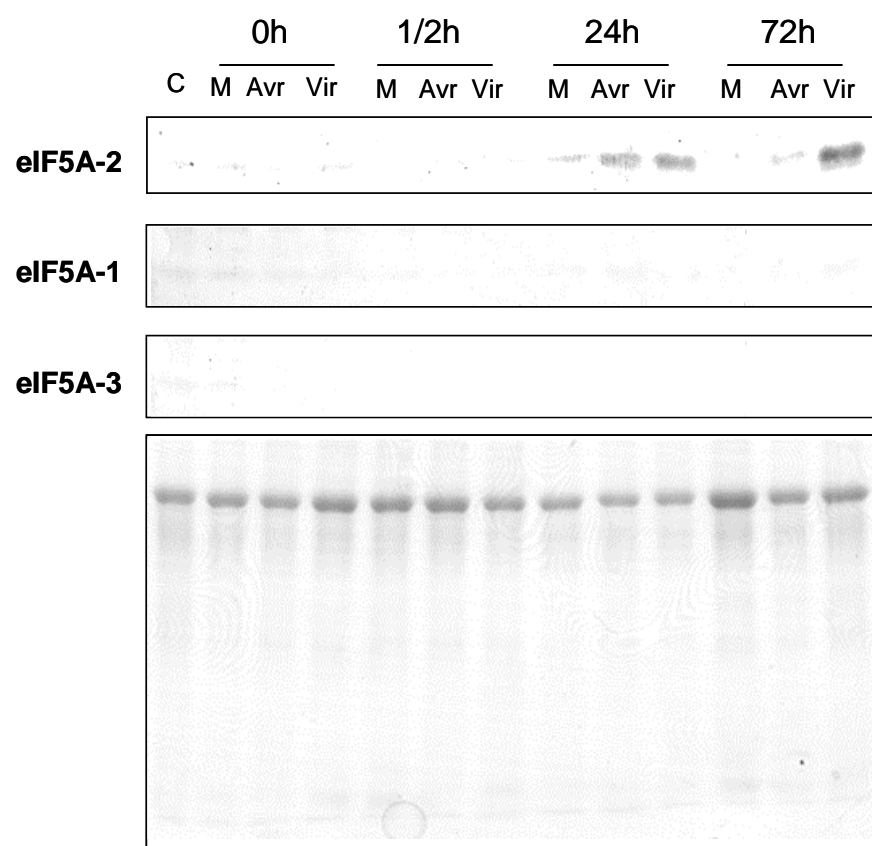


Figure 3. Western blot analysis comparing the expression of eIF5A isoforms in response to pathogen ingress in wild type *Arabidopsis*.

Protein was isolated from the leaves of 4-week-old plants inoculated with either 10 mM MgCl₂ solution (Mock), an avirulent (*Avr*), or a virulent strain (*Vir*) of *Pst* DC3000. The tissue was harvested immediately after the inoculation, 30 minutes after, 24 h after, and 72 h post inoculation. The SDS-PAGE gel stained with Coomassie blue is shown for each lane as a loading control.



To ensure that the disease symptoms observed in plants inoculated with virulent bacteria correlated with an increase in bacterial population within the leaf tissue, *P. syringae* had to be quantified. The classic phytopathological technique for quantifying bacterial virulence is an assay measuring bacterial multiplication within the host tissue (Katagiri et al., 2002). Virulent pathogen *Pst* DC3000 inoculated at low concentrations of 1×10^6 cfu/ml can colonize the host tissue and in the course of several days multiply more than 10,000-fold within the host tissue (to a level of 1×10^8 cfu/leaf disk). In contrast, the avirulent strains of *Pst* DC3000 carrying the *avrRpt2* gene in the same time course will either not multiply significantly or grow only 10- to 100-fold within the host tissue. The massive multiplication of the virulent bacteria correlates well with symptom development. Specifically, the bacterial strain attains the maximal population immediately in advance of significant symptom development which, in the case of *Pst* DC3000 infection, is characterized by necrotic lesions surrounded by diffuse chlorosis. The avirulent strains do not multiply to high populations and also do not produce disease symptoms.

In the present study, the inoculated tissue was assayed for bacteria at regular intervals, including the day of inoculation to establish the bacterial level immediately following inoculation. The levels of virulent bacteria were compared to the levels of avirulent *Pst*, and the results were plotted as culturable bacterial number per leaf disk against time. This is illustrated in Figure 4. The bacterial level immediately after inoculation was comparably low for both virulent (*Pst* DC3000) and avirulent (*Pst* DC3000 *avr*) strains (Fig. 4). However, a difference in growth was detectable at 24 hours; with the avirulent strain multiplying to 8.3×10^4 cfu per leaf disk and remaining at that level through 72 h (Fig. 4). In contrast, the virulent strain was able to multiply to 5.1×10^5 cfu, which is nearly three logs higher, by 24 hours and up to 10^7 cfu per leaf disk by the end of the assay at 72 hours (Fig. 4). Typical phenotypic manifestations of infection by virulent and avirulent strains of *Pst* DC3000 at 72

Figure 4. Virulent (Pst DC3000) and avirulent (Pst DC3000 *avr*) *Pseudomonas syringae* levels within wild type *Arabidopsis* leaves.

Plants leaves were pressure infiltrated with 1×10^6 cfu/ml bacterial suspension using a syringe. Bars represent mean log colony forming units (cfu) per leaf disk 4mm in diameter \pm sample SD.

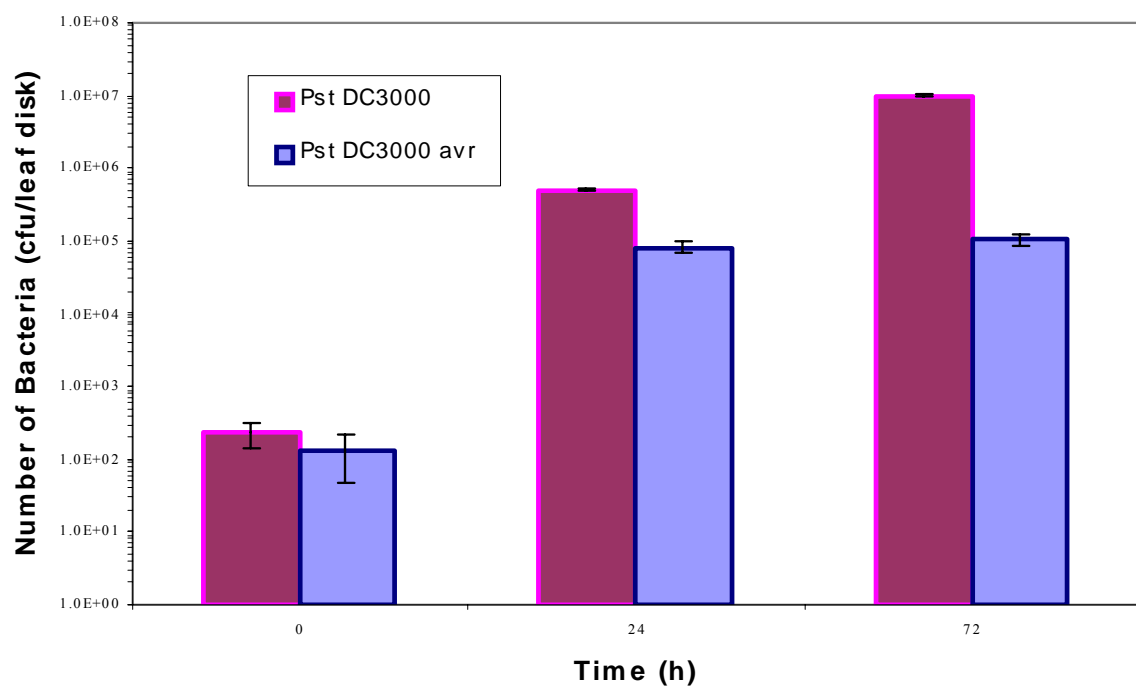
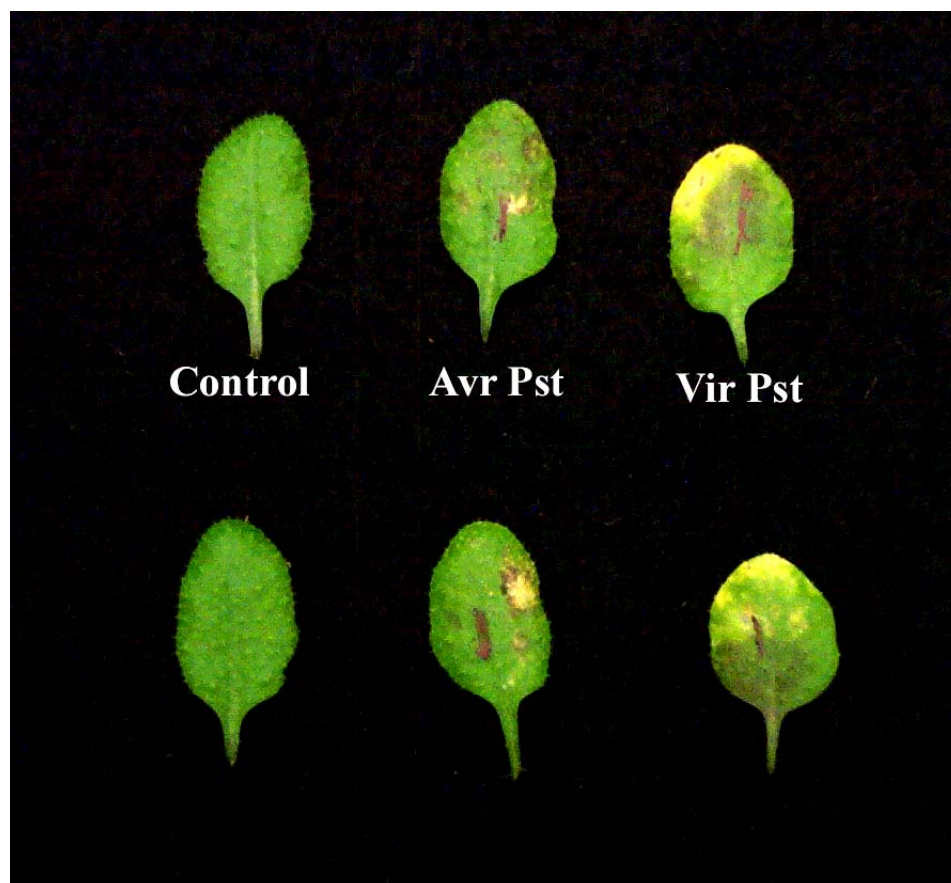


Figure 5. Photograph illustrating disease and HR symptoms in *Arabidopsis* leaves caused by virulent and avirulent strains of *Pst* DC3000, respectively.

Leaves were inoculated with 1×10^6 cfu/ml of bacteria, and the picture was taken 72 hours after inoculation. The untreated control leaves are shown on the left.



hours post-inoculation are illustrated in Figure 5. The necrotic, dark-colored area surrounded by extensive chlorosis is the characteristic appearance of a ‘speck’ disease caused by the virulent strain of *Pst* (Fig. 5). The HR caused by the avirulent strain appears as small dry lesions (Fig. 5) that develop within 24 hours after infection.

3.1.2 Immunolocalization of eIF5A-2 in *Arabidopsis* leaves infected with *Pst* DC3000

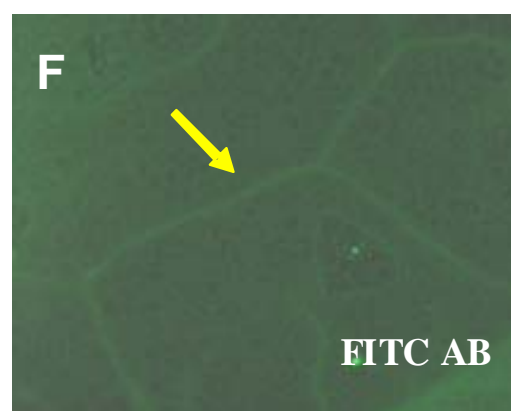
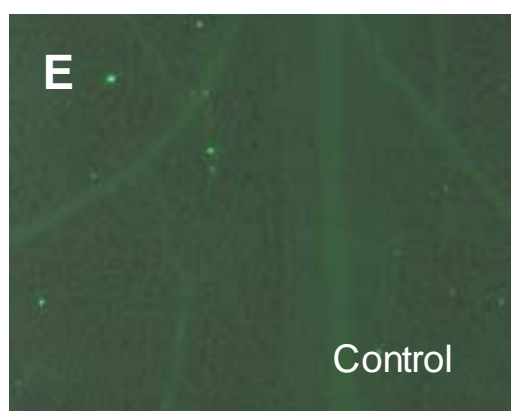
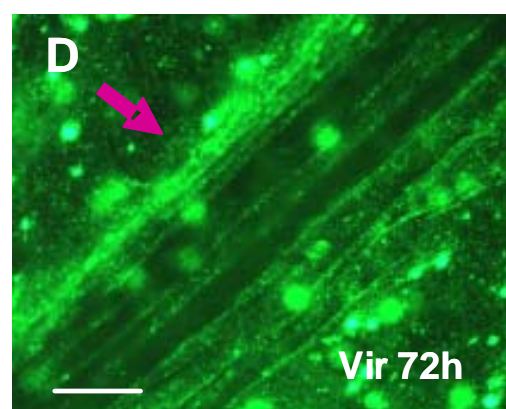
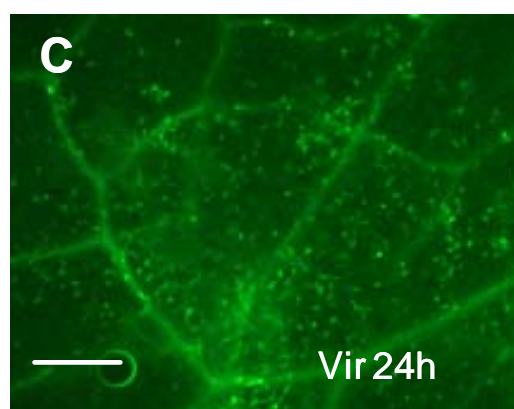
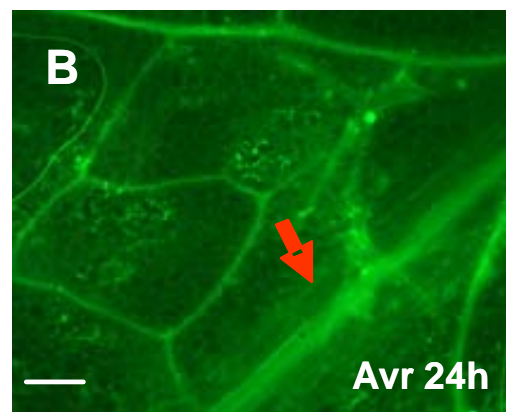
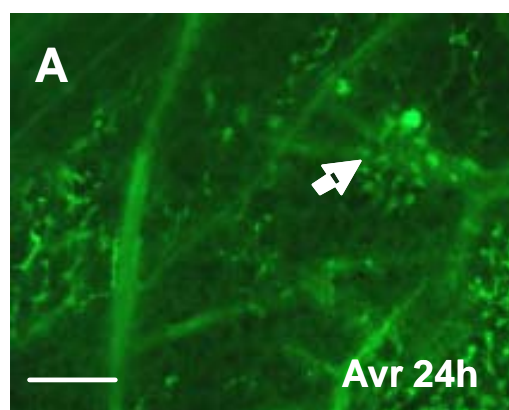
To be able to fully characterize the expression of a given gene, it is imperative to determine the location of the expression within the organism. To complement the information gathered from Western blots of total protein analysis, the infected *Arabidopsis* leaf tissue was subjected to immunohistochemical analysis to visualize the pattern of eIF5A-2 accumulation during pathogen ingress. Fixed and cleared disks excised from leaves of *Pst*-infected *Arabidopsis* were incubated with purified eIF5A-2 primary antibody and fluorescein-conjugated secondary antibody, which enables *in situ* visualization when the samples are viewed by fluorescence microscopy. Figures 6A and B demonstrate that accumulation of eIF5A-2 protein is restricted to cells of plants treated with avirulent bacteria. In leaves treated with virulent bacteria, however, there is much more extensive accumulation of eIF5A-2 protein, particularly in regions near the midvein (Figs. 6C and D). The autofluorescence of the xylem is visible in the control and 2nd antibody-treated tissue (Figs. 6E and F).

3.1.3 Expression profiles of eIF5A-2 in infected plants grown under short- and long-day conditions

Arabidopsis, a facultative long-day plant, when grown under short-day conditions exhibits delayed flowering and more robust rosettes with an increased number of leaves. This phenotype is preferred in *Arabidopsis* – *P. syringae* studies because the enlarged leaves allow for more accessible infiltration. However, the 9-hour light regime has been linked to increased lignifications and stress-like responses in *Arabidopsis* (Weretilnyk et al., 1995). To study the impact of changes in the circadian rhythm on eIF5A-2 expression induction, the expression profiles of eIF5A-2 in long- and

Figure 6. Immunolocalization of eIF5A-2 protein in wild type *Arabidopsis* leaf tissue treated with the virulent and avirulent strains of *Pst*.

Images were obtained with a Zeiss inverted microscope Axiovert 200 equipped with a FITC filter. Antibody binding was visualized with FITC-conjugated secondary antibody. Arrows identify specific immunostaining. A. Close up view of tissue infected with avirulent *Pst* 24 hours post infiltration. White arrow points out a cluster of cells expressing eIF5A-2 near a minor vein of the leaf. Bar = 0.5mm. B. Tissue 24 hours after inoculation with avirulent *Pst*. Red arrow represents immunostained major vein. Bar = 1mm. C. Tissue inoculated with virulent strain of *Pst* 24 hours post infiltration. Bar = 1mm. D. Close up of tissue treated with virulent strain of *Pst* 72 hours post inoculation. Purple arrow represents localization of eIF5A-2 within the cells in close proximity to the midvein. Bar = 100µM. E. Immunostaining of untreated control tissue. Bar = 0.5mm F. Tissue treated with virulent bacteria incubated with FITC-conjugated secondary antibody only to assess background immunostaining and autofluorescence. Bar = 0.5mm.



short-day-grown plants were examined. Figure 7 demonstrates that eIF5A-2 accumulation in plants grown under short light cycles is initiated earlier than in long-day plants, commencing at 24 hours post inoculation and increasing slightly at 72 hours. By contrast, in long-day plants there is no detectable expression at 24 hours post-infection, but the level of expression at 72 hours post-inoculation is more pronounced than for short-day plants (Fig. 7). The lower expression of eIF5A-2 at 72 hours in the short-day plants presumably reflects slower development of infection and may be attributable to a smaller initial inoculum because of a decrease in substomatal chamber volume caused by the higher level of lignification as well as a decrease in the apoplastic volume, both of which accommodate the inoculum.

It has also been proposed that *Pst* may undergo a short biotrophic phase within the apoplast, where the pathogen feeds on the small supply of nutrients present, before initiating its necrotrophic phase (personal communication from Dr. P. Goodwin, University of Guelph). This biotrophic phase may be shorter in duration in plants with increased lignification in which case the necrotrophic phase would commence earlier. This in turn could account for the earlier initiation of increased eIF5A-2 expression in short-day plants. When bacterial numbers were examined in short- and long-day plants, the assays revealed that the population size was indeed lower in plants grown under the short-day regime (Fig. 8). The slower population growth in short-day plants in addition to the stronger general resistance provided by enhanced lignification presumably allowed for a more rapid rise in specific pathogen resistance response, slowing the systemic spread of the pathogen.

Figure 7. Western blot analysis of total protein isolated from virulent *Pst*-infected leaves of wild type *Arabidopsis* plants grown under long and short light regimes.

Plants were maintained under either short (9h light/15h dark) or long (16h light/8h dark) day photoperiods for 4 weeks and then selected leaves were pressure-infiltrated with 1×10^6 cfu/ml bacterial suspension. The total protein was probed with eIF5A-2 antibody. The corresponding SDS-PAGE gel stained with Coomassie blue is included as a loading control.

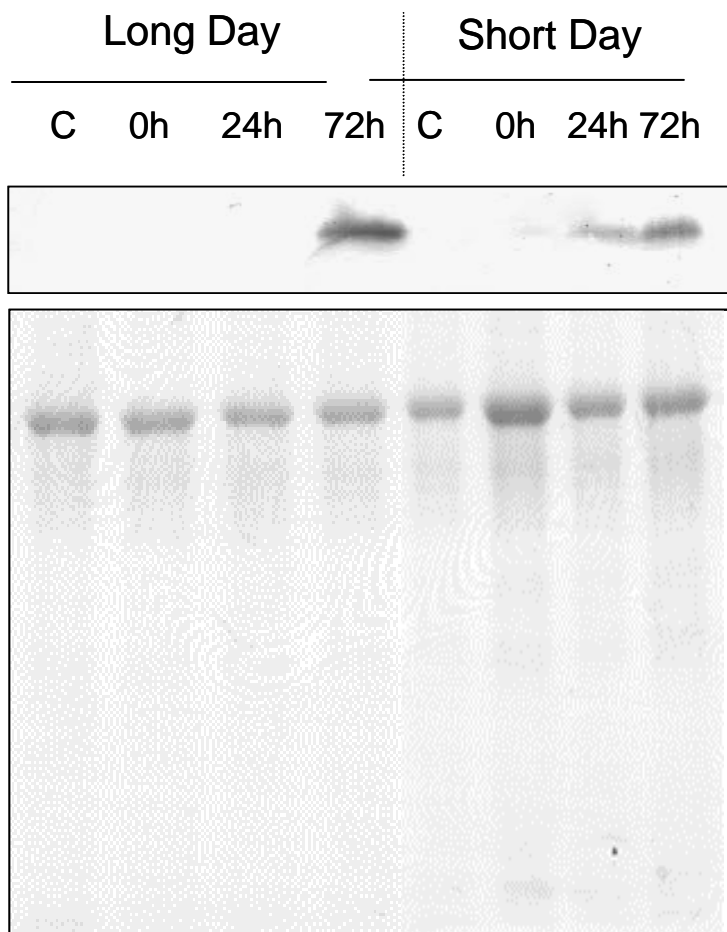
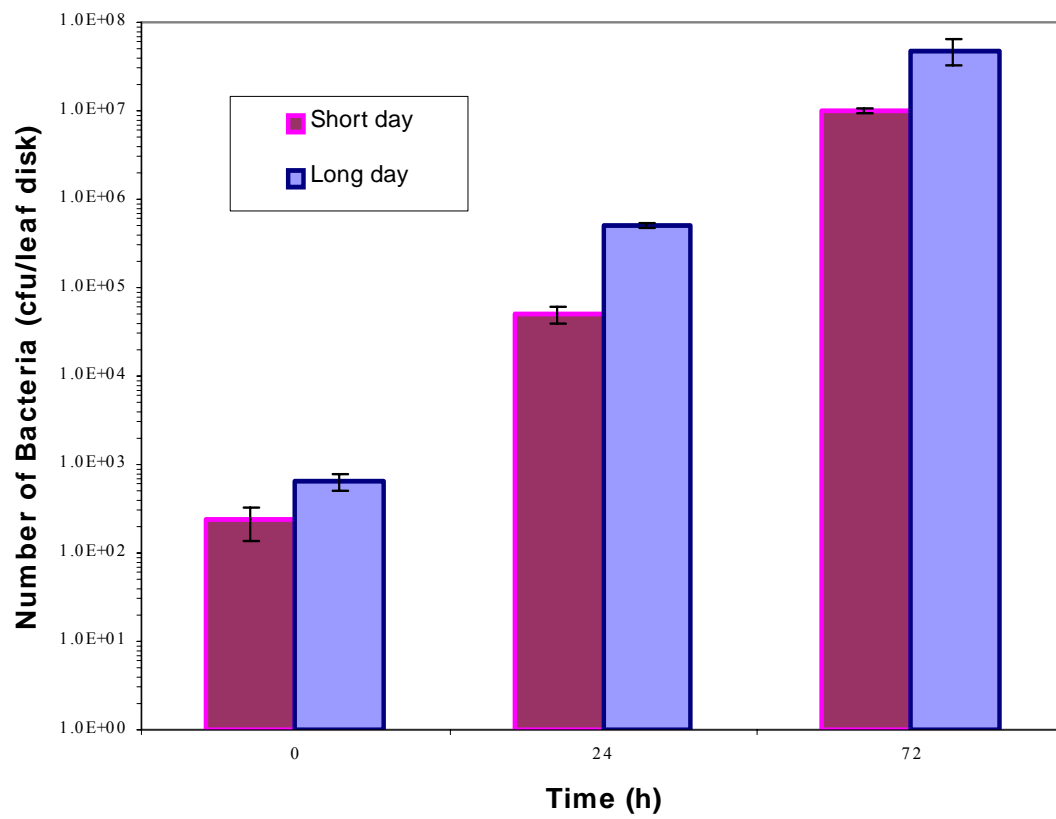


Figure 8. Virulent *P. syringae* levels within the leaves of wild type *Arabidopsis* plants grown under long-day and short-day light regimes.

Plants were maintained under either short (9h light/15h dark) or long (16h light/8h dark) day photoperiods for 4 weeks and then selected leaves were pressure-infiltrated with 1×10^6 cfu/ml bacterial suspension. Bars represent mean colony-forming units (cfu) per leaf disk 4mm in diameter \pm sample SD.



3.2 Expression Analysis of eIF5A in Response to Wounding

Wounding causes extensive changes in the pattern of defense protein synthesis leading to localized resistance at the site of lesion (Belenghi et al., 2003). It is suspected that mechanical wounding also triggers host cell death around the lesion as an initial response. To investigate whether eIF5A-2 is also involved in the wounding response in *Arabidopsis*, leaves of plants were mechanically wounded using a hemostat, and the expression of eIF5A-2 was analyzed through Northern and Western blotting.

Similar to pathogen-induced expression, wounding of 4-week-old *Arabidopsis* leaves resulted in transcription of the eIF5A-2 isoform, 0.7 kb in size, as shown by Northern blot analysis (Fig. 9). The transcript was evident at 0 h and showed a steady increase in abundance up to 5 hours post-wounding, confirming the constitutive expression of the eIF5A-2 gene seen with the pathogenesis response. The up-regulation of eIF5A-2 protein was also apparent from Western blot analysis (Fig 10). The expression of the 18 kD protein was evident at 4 h post wounding. The levels went up between 4 and 7.5 hours and remained elevated at 10 hours after wounding (Fig. 10). Again, as for pathogen ingress, up-regulation of the eIF5A-2 protein is achieved post-transcriptionally (Figs. 9 and 10).

Figure 9. Northern blot analysis of total RNA isolated from the rosette leaves of 4-week old *Arabidopsis thaliana*, wounded with a hemostat.

The RNA blot was probed with radiolabelled 3'UTR eIF5A-2. Lanes 0h through 5 h represent harvesting times of the leaves after wounding. The ethidium bromide stain of rRNA is shown as a loading control.

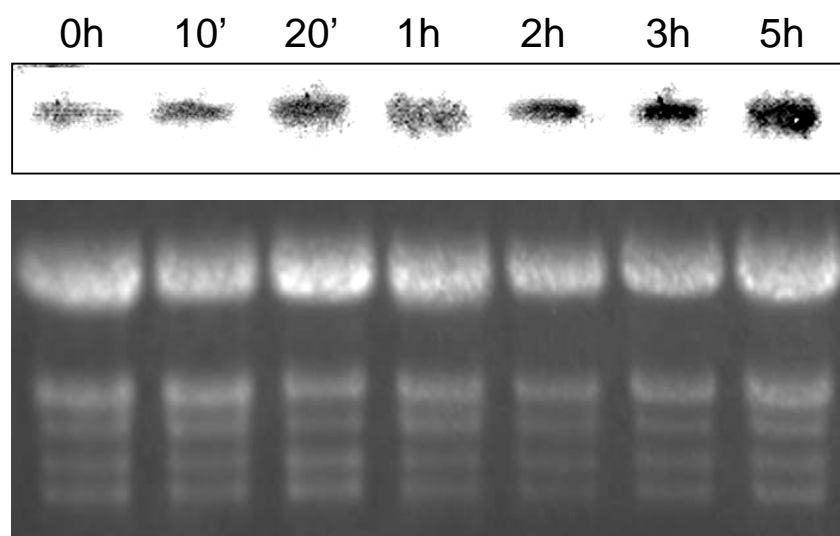
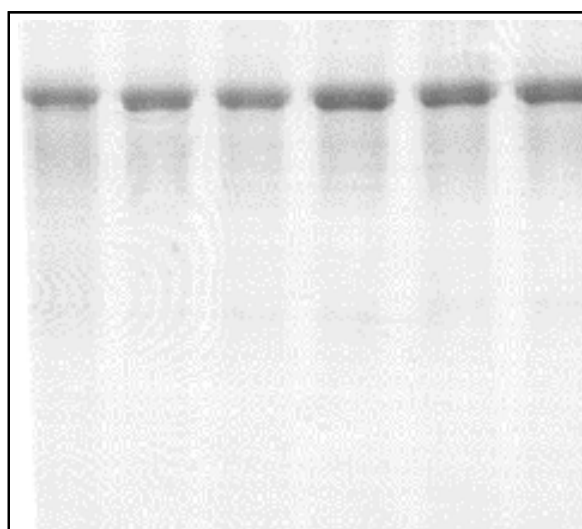


Figure 10. Western blot analysis showing the expression of eIF5A-2 isoform in response to wounding of wild type *Arabidopsis* leaves.

Protein was isolated from the rosette leaves of 4-week-old plants. The tissue was harvested immediately after wounding, 4 h after, 7.5 h, 9 h, and 10 h post- wounding. Control plants were left untreated. The SDS-PAGE gel stained with Coomassie blue is shown for each lane as a loading control.

C 0h 4h 7.5h 9h 10h



3.3 Analysis of Antisense eIF5A-2 Transgenic Plants

3.3.1 Production of transgenic antisense 3'UTR eIF5A-2 plants

To assess the impact of eIF5A-2 protein on signal transduction during pathogen ingress, the expression of eIF5A-2 was suppressed by expressing the gene in the antisense orientation under the regulation of a constitutive promoter. The 3'-untranslated region (UTR) of *Arabidopsis* eIF5A-2 cDNA, used as the transgene, was amplified by PCR using specific primers designed by Marianne Hopkins in Dr. J. E. Thompson's laboratory based on the sequence available in GenBank. The obtained PCR product was 208 bp in length and included *SacI* and *XhoI* restriction enzyme sites to facilitate cloning into pKYLX71.

The binary vector, pKYLX71, containing the 3'-UTR eIF5A-2 in antisense orientation under the regulation of a double 35S constitutive cauliflower mosaic virus promoter (Fig. 11), was electroporated into *Agrobacterium tumefaciens* C58, and flowering *Arabidopsis thaliana* plants were transformed with the *Agrobacterium* by vacuum infiltration (Bechtold et al., 1998). The primary transformants T₀ were allowed to mature and set seed in a growth chamber. T₁ seedlings were selected by growth on kanamycin-containing medium (Fig. 12A) and grown to maturity. T₂ and T₃ seedlings were similarly selected on kanamycin-containing media and grown to maturity. Seeds from T₃ plants that gave rise to 100% kanamycin-resistant seedlings were considered homozygous (Fig.12B).

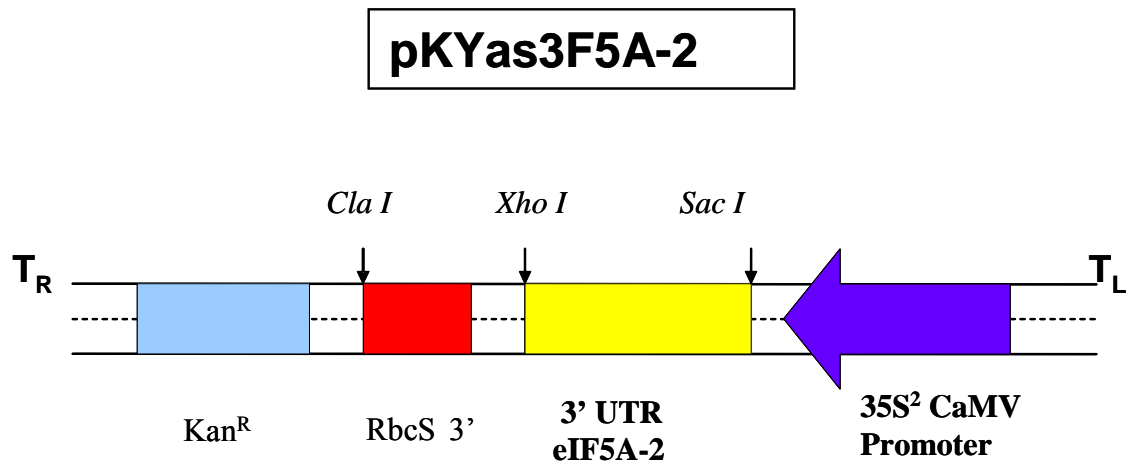
3.3.2 Screening of transgenic plants

Leaves of homozygous T₃ plants were screened for down-regulation of eIF5A-2 protein using infection with a virulent strain of *Pst*. Four-week-old wild type and transgenic plants were inoculated with *Pst* DC3000 at 1x10⁶ cfu/ml. The total leaf protein was isolated and analyzed by Western blot analysis. Protein levels of eIF5A-2 from 9 transgenic lines were compared to the levels in wild type

Figure 11. Antisense construct used to transform *Arabidopsis*.

A. Schematic diagrams of the construct *pKYas3F5A-2*. The PCR product amplified from the 3' untranslated region of the cDNA was inserted into pGEM. The recombinant pGEM vector was cut with *SacI* and *XhoI*, and the insert was ligated into a similarly cut binary vector, pKYLX71. This process gave the antisense orientation of 3'UTR *eIF5A-2* under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Kan^R, kanamycin-resistance gene; TR and TL, right and left border of T-DNA, respectively. B. An agarose gel of double-digested plasmid from 4 colonies of *E. coli* DH5- α transformed with pKYas3F5A-2. Lanes: 1, 2, 3, and 4, recombinant plasmid isolated from four different transformant colonies and double-digested with *Xho I* and *Sac I* restriction enzymes; lane 5, DNA Molecular Weight Marker XIV (Roche); lane 6, DNA Molecular Marker X (Roche). The expected size of the cDNA insert is 202 bp.

A.



B.

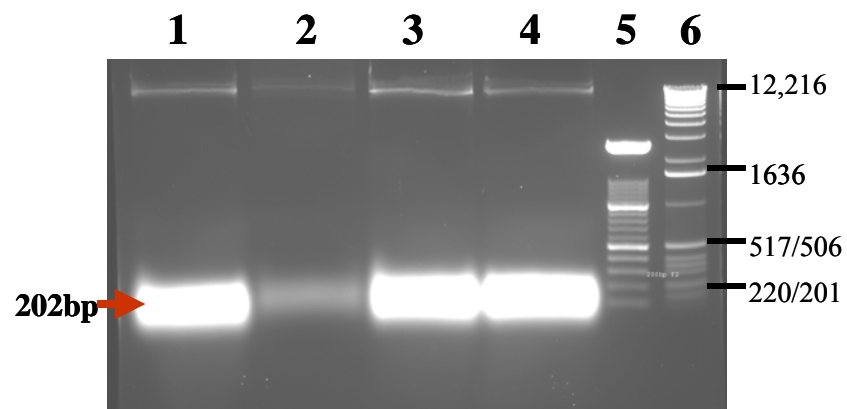
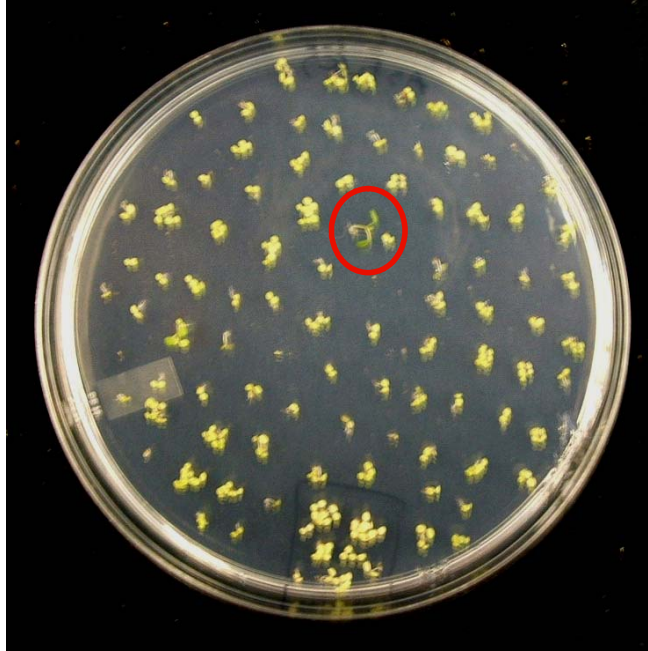


Figure 12. Segregation analysis of transgenic plants.

A. Photograph of a half-strength MS kanamycin (50 µg/ml) selection plate used to screen T₁ as3'UTReIF5A-2 seeds. Enclosed within a circle is a green successfully transformed plant, which is able to grow on the selection plate due to retention of the chimeric transgene containing kanamycin resistance. B. Photograph illustrating 100% survival of T₃ transgenic line 8A grown on a half-strength MS kanamycin (50 µg/ml) selection plate.

A



B



and binary plants, which were previously found to show strong up-regulation of eIF5A-2 at 72h post inoculation with *vir Pst* (Fig. 13). Lines designated as 8A, 8C, 8E, 8G, and 13D showed significant down-regulation in eIF5A-2 expression. The levels of eIF5A-2 in lines 1C and 1D were comparable with the levels in wild type plants (Fig. 13).

Population growth of *Pst* DC3000 in the leaves of wild type and transgenic plants over the course of time is illustrated in Figure 14. Consistent with the earlier experiments, wild type plants showed a 5-log increase in bacterial population 72 hours post-inoculation; binary plants supported similar bacterial growth, whereas the transgenic line 8A proved to have the lowest levels of pathogen growth, with a 99% decrease by comparison with wild type plants. Furthermore, lines 8A and 8C were shown to be impervious to chlorophyll degradation associated with disease establishment as demonstrated in Figure 15. Of note as well is that the antisense eIF5A-2 transgenic *Arabidopsis* plants grew, flowered, and set seed in a manner similar to wild type *Arabidopsis*, with no detectable morphological or physiological alterations (Fig.17).

The infected wild type and transgenic *Arabidopsis* tissue was subjected to immunolocalization analysis to compare the pattern of eIF5A-2 accumulation during pathogen ingress. Fixed and cleared disks, excised from leaves of *Pst*-infected *Arabidopsis*, were incubated with purified eIF5A-2 primary antibody and fluorescein-conjugated secondary antibody, which provides *in situ* visualization when the samples are viewed by confocal fluorescence microscopy. Figure 18A demonstrates that FITC immunoreactivity was clearly detectable within the cytoplasm of dying mesophyll cells of wild type leaves (Fig. 18A). The eIF5A-2 protein appears to be pushed against the cell wall by the vacuole in the centre (Fig. 18A). By contrast, the localized accumulation of eIF5A-2 protein around a stomate illustrated for plants of the transgenic line 8A in Figure 18B indicates contained infection by *Pst*, which was pressure-infiltrated through the stomate. Thus it would appear that, for the transgenic plants, the bacteria were not able to spread beyond the substomatal chamber into which they were

Figure 13. Western blot analysis of total protein isolated from wild type and transgenic *Arabidopsis* leaves infected with virulent strain of *Pst* 72 hours post inoculation.

Protein was probed with eIF5A-2 antibody. The SDS-PAGE gel stained with Coomassie blue is shown for each lane as a loading control.

WT Bin 8A 8C 8D 8E 8G 1C 1D 13D 9A

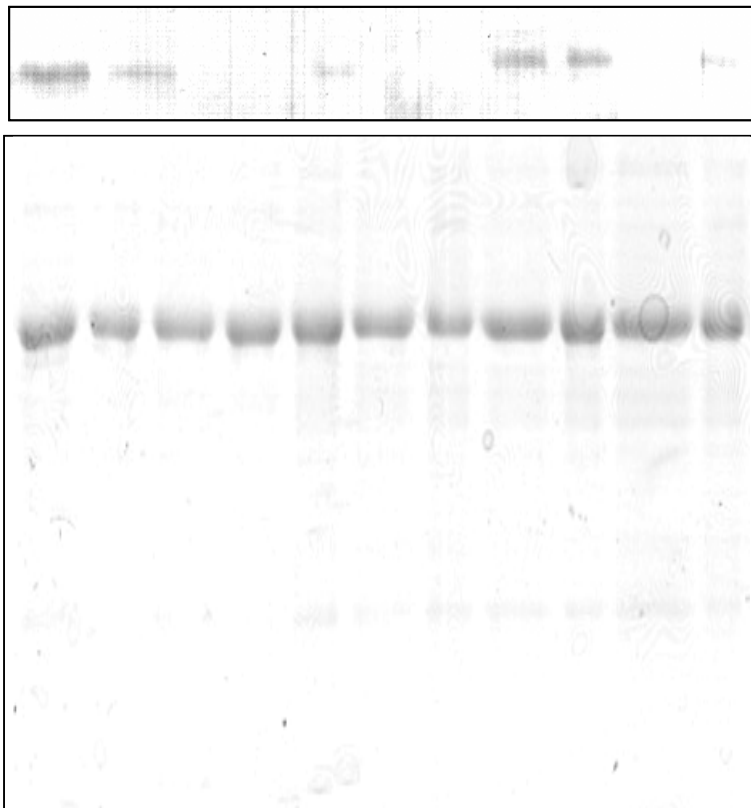


Figure 14. Virulent *Pst* levels within wild type and transgenic *Arabidopsis* leaves.

The leaves were pressure-infiltrated with 1×10^6 cfu/ml bacterial suspension. Bars represent means \pm SD.

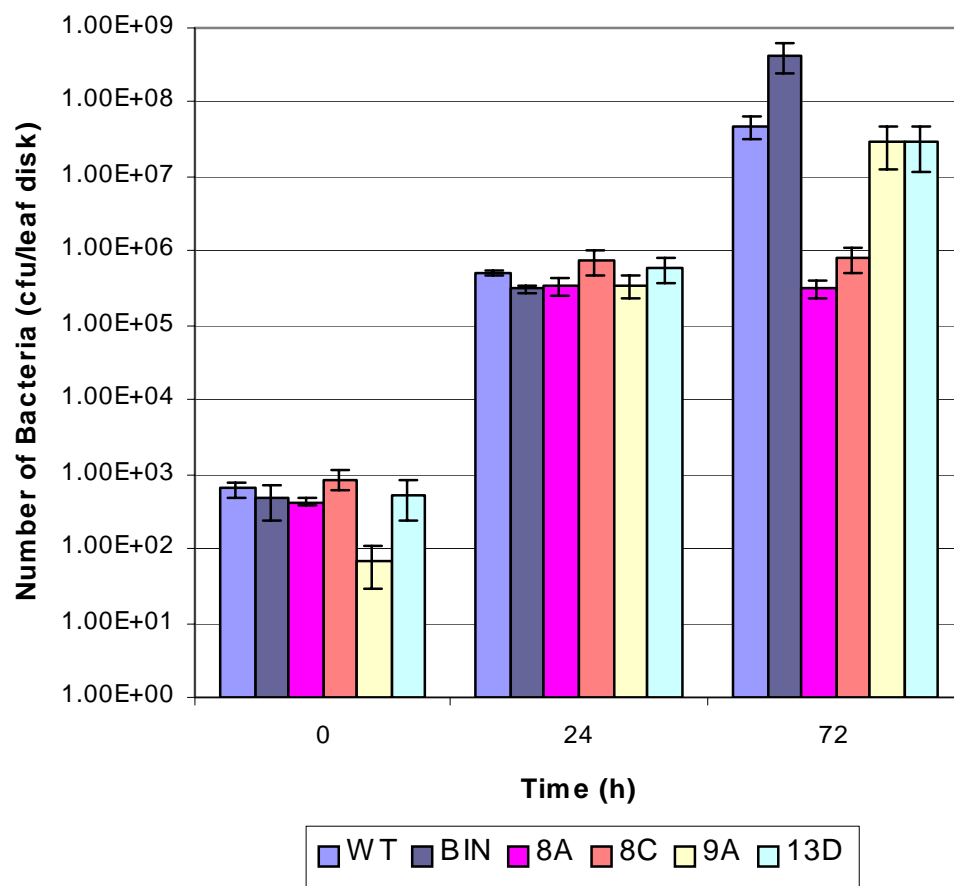


Figure 15. Chlorophyll levels in the leaves of wild type and transgenic *Arabidopsis* plants 72 hours post infection with virulent strain of *Pst*.

Control wild type plant was left untreated.

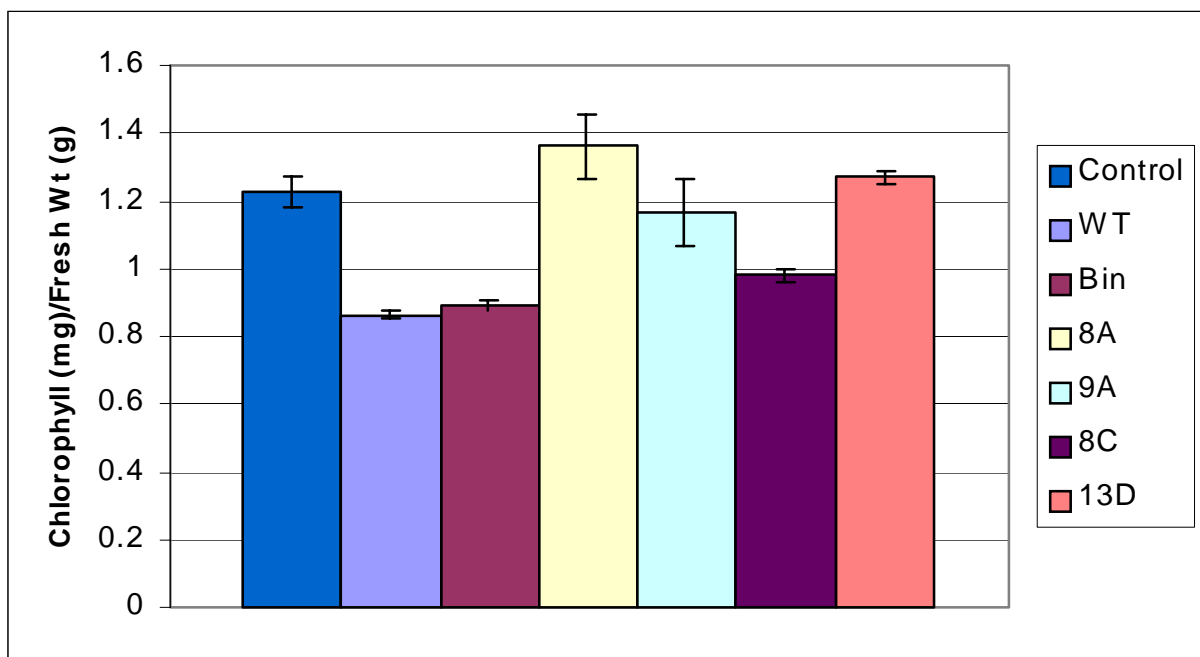


Figure 16. Photograph of wild type and transgenic *Arabidopsis* plants 72 hours post inoculation with virulent strain of *Pst* and MgCl₂ (Mock).

Full rosettes with marked inoculated leaves are shown in the left and central panels. Individual leaves of wild type, binary and transgenic 8A plants showing various degrees of infection are shown in the right panel.

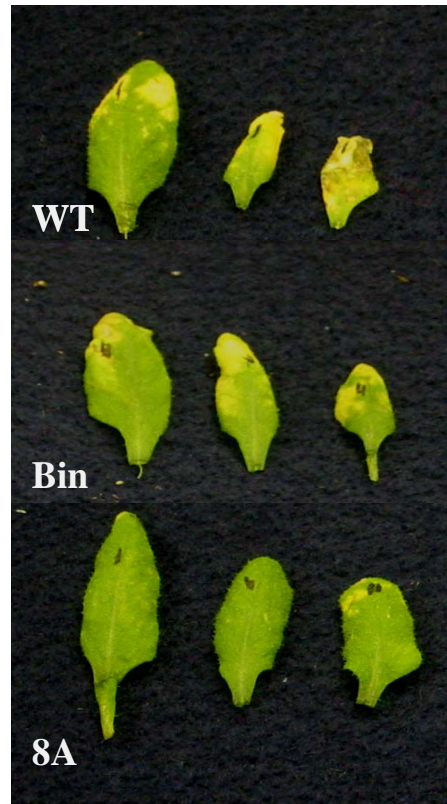
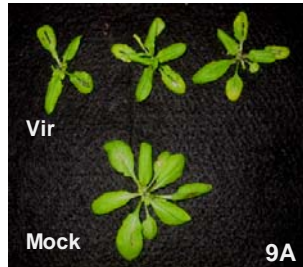


Figure 17. Photograph illustrating similarity in phenotype observed between wild type and generation T3 transgenic lines 4 and 5 weeks after planting.

8A, 8C, 8G, and 8F represent sister transgenic lines.

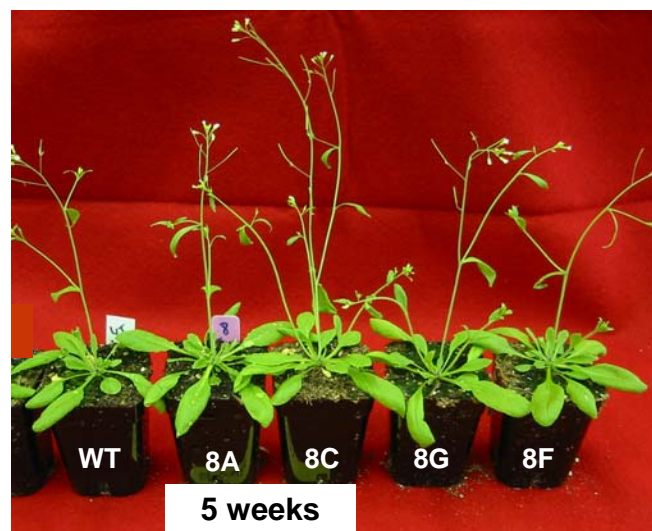
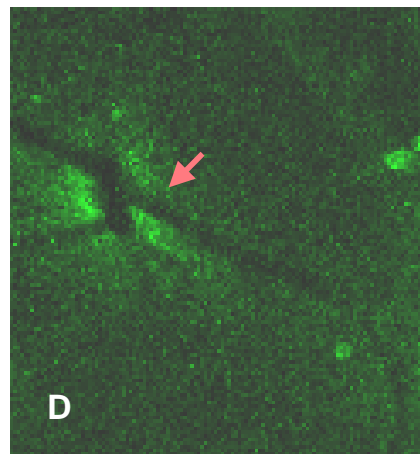
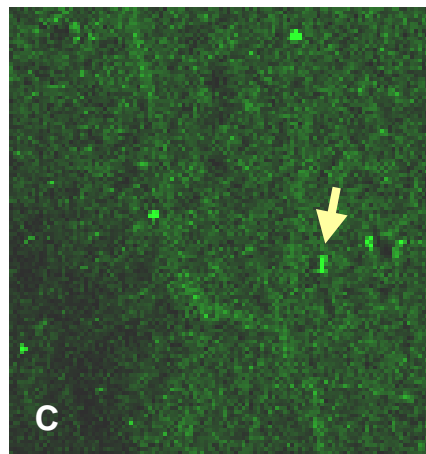
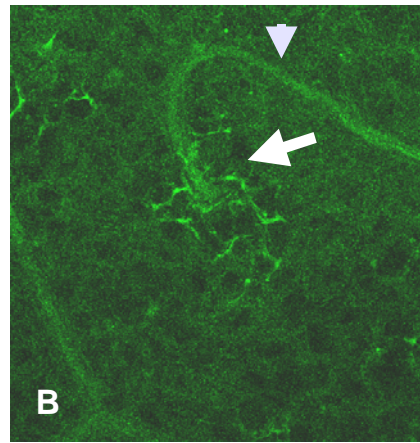
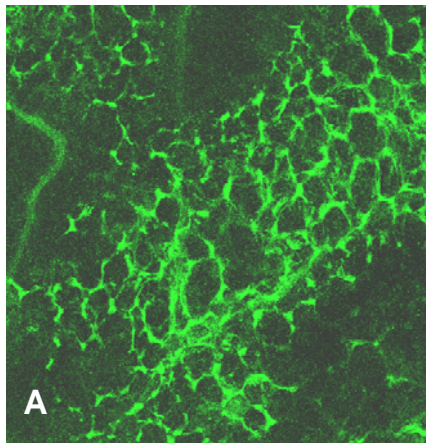


Figure 18. Immunolocalization of eIF5A-2 protein in wild type and transgenic *Arabidopsis* leaf tissue treated with virulent strain of *Pst* 72 hours post inoculation.

Confocal images were obtained with a Zeiss 510 laser scanning confocal microscope. Antibody binding was visualized with FITC-conjugated secondary antibody. Arrows identify specific immunostaining. A. Treated wild type *Arabidopsis*. B. Treated transgenic line 8A. Purple arrowhead shows staining of the minor vein. White arrow shows a stomate and localized eIF5A-2 expression around it. C. Control wild type. Yellow arrow shows localization of FITC inside the substomatal space. D. Control transgenic line 8A. Pink arrows indicate a trichome with unspecific binding of FITC.



delivered due to the lack of nutrients required for multiplication. However, some expression of eIF5A-2 was observed (Fig. 18B). Indeed, slight accumulation of eIF5A-2 in the phloem cells of the vascular system of transgenic leaves may indicate that the protein is phloem-mobile (Fig. 18B). Thus during infection, bacteria may rely on the host's phloem system to transport the host cell death signals systemically.

The lack of availability of eIF5A-2 for initiation of the host cell death pathway may interfere with bacterial ability to cause release of plant nutrients into the apoplast. The slowdown in the infection process due to lack of nutrients may give the host enough time to mount a general resistance response allowing for localized and systemic accumulation of defense proteins.

Discussion

Throughout history, plant pathogens have plagued humans by damaging crops grown for food and in many cases, in combination with other factors, have led to the starvation and death of millions of people. Some commonly known examples are the famines that resulted from the *Phytophthora* late blight epidemic of potato in 1845-1846 in Ireland and the *Cochliobolus* brown spot epidemic of rice in Bengal in 1943. In addition to the loss of human life, plant diseases cost millions of dollars in crop losses each year. In North America, the inadvertent introduction of exotic pathogens through free trade has become a great concern. For example, the introduction of the chestnut blight pathogen, *Cryphonectria parasitica*, from Europe in 1904 effectively eliminated the American chestnut from North America (Griffin et al., 2004). Similar potential damage is currently occurring with disease epidemics of forest trees including sudden oak death caused by *Phytophthora ramorum* (Linderman et al., 2002) and pitch canker of pine caused by *Fusarium circinatum* (Chimwamurombe et al., 2001).

The exact amount of loss is extremely difficult to estimate because our ability to measure loss is limited by sampling methods and the incidence of disease in crop fields. Incremental costs include, among others, the added cost of quality control or harvesting and processing time. The problem is compounded by insufficient and costly methods of controlling plant-pathogens through conventional methods such as pesticides. Indirect costs of using pesticides may actually outweigh the perceived benefits of using these toxic compounds. A 1992 study of pesticide costs in the US (Pimentel et al., 1992) reveals that pesticides cost at least \$8 billion per year. Costs include many factors: the loss of fish, bird, wildlife, and natural predators of pests; increased resistance of pests to pesticides, and public health effects. It was estimated that \$787 million is spent on the 67,000 poisonings and 27 deaths in the United States annually. These figures plus the extent of potential plant disease associated with agricultural crops are compelling arguments for controlling plant disease.

A significant amount of crop research around the world is engaged in genetic engineering in attempts to increase resistance to plant pathogens in order to prevent crop losses and reduce pesticide usage. Although a large number of pathogen-induced genes has been cloned and studied in detail at the molecular level, very little is known about the signal transduction pathways that lead to their activation. Therapeutic modification of gene expression or signaling pathways involved in programmed cell death is also a rapidly expanding area in animal and human health research.

Host cell death is one of the consequences of plant–pathogen interactions. It can lead to either disease resistance termed systemic acquired resistance (SAR) (Greenberg et al., 1994, Mittler et al., 1995, Bendahmane et al., 1999) or necrotic disease (Wang et al., 1996, Navarre and T.J., 1999). SAR usually results from a response to incompatible pathogens and symptomatic cell death during the hypersensitive response (HR), which is elicited by specific recognition of pathogen-derived molecules from avirulent pathogens by plant proteins encoded by resistance (*R*) genes. Recognition is followed by local accumulation of reactive oxygen species (ROS), nitric oxide (NO), and salicylic acid (SA), all of which potentiate resistance and pro-death pathways (Shirasu and Schulze-Lefert, 2000). HR is accompanied by fortification of cell walls and accumulation of pathogenesis-related (PR) mRNAs and antimicrobial phytoalexins. These responses serve to protect the plant by restricting the growth of pathogens. Necrotic disease is usually caused by compatible pathogens that use dead tissue as a nutrient source. The cells are either killed by the action of pathogen-derived toxins or are induced to die at a late stage of infection. In the plant-pathogen system, these two types of cell death are readily separated by the speed, amplitude, and localization of activation (Brodersen et al., 2002).

The mechanistic basis of this cellular disruption and consequent death is complex and poorly characterized, but it is established that host responses to pathogens are dependent on gene expression, involve signal transduction, and require energy (Katagiri et al., 2002). In animals, a genetically regulated, signal transduction-dependent programmed cell death process, commonly referred to as

apoptosis, is conserved over a wide range of phyla. The basic function of apoptosis is to direct the selective elimination of certain cells during development, but it also is a master template that is involved in host responses to many pathogens.

Programmed cell death in plants, while widely observed, has not been studied extensively at either the biochemical or genetic level. Current data suggest that activation or suppression of programmed cell death may underlie diseases in plants as it does in animals (Dickman et al., 2001, Mittler et al., 1997). Specifically, transgenic tobacco expressing animal antiapoptotic transgenes such as Bcl-2, Bcl-xl, and CED-9, developed heritable resistance to several necrotrophic fungal pathogens, suggesting that disease development required host cell death pathways (Dickman et al., 2001). Recently, Ausubel and colleagues (Stone et al., 2000, Asai et al., 2000) have shown that treatment of *Arabidopsis* protoplasts with the pathogen toxin, fumonisin B1, results in terminal deoxynucleotidyltransferase-mediated UTP end labeling, a well established index of apoptosis, and also that toxin infiltration of intact plants resulted in cell death, generation of reactive oxygen intermediates, and other biochemical markers associated with plant defense responses. These authors also suggested that toxin-mediated PCD might be important during successful plant infection by pathogens.

To date, no plant homologues of caspases or Bcl-2-like proteins have been identified, although caspase-like activity has been detected (del Pozo and Lam, 1998). Further, cysteine proteases are important in a number of plant PCD situations, including senescence (Drake et al., 1996, Xu and Hanson, 2000), xylogenesis (Minami and Fukuda, 1995), and oxidative stress (Solomon et al., 1999). It is possible that functional analogs may be obscure at the sequence level, which is consistent with the divergence of plant and animal species. However, there is growing circumstantial evidence that similar pathways exist in plants and animals, but the extent of the overlap is not yet resolved.

Recent efforts to further understand the animal apoptotic pathway have lead to recognition of human eukaryotic translation initiation factor 5A (eIF5A) as a factor promoting programmed cell death in human umbilical vein endothelial cells (HUVEC) (Lee et al., 2002). These authors reported that blocking hypusination of eIF5A by inhibiting DHS can protect HUVEC cells from serum-starvation-induced apoptosis, indicating that hypusinated eIF5A is required for apoptosis. In light of this and other similar reports, it is conceivable that plant eIF5A may act in an analogous way by promoting programmed cell death in plant cells.

The present study was undertaken to help elucidate the role of eIF5A during cell death in response to wounding and pathogen attack in *Arabidopsis thaliana*. eIF5A is proposed to act as a nucleo-cytoplasmic shuttle protein, which specifically binds newly transcribed suites of mRNA and transports them out of the nucleus into the cytoplasm for translation. Data obtained in the present study show that the eIF5A-2 isoform in *Arabidopsis* is expressed in response to pathogen attack and mechanical wounding (Figs. 3 and 10). A unifying aspect of these results is the fact that both treatments result in host cell death. In wounding, the cells at the site undergo decompartmentalization, release of stored materials and dehydration leading to localized necrosis (Leon et al., 2001). In pathogenesis, the cells undergo rapid PCD associated with HR, or slower PCD induced by a necrotizing pathogen (Katagiri et al., 2002). The eIF5A-2 isoform was constitutively expressed at the RNA level, however the expression at the protein level appeared to be more controlled, suggesting post-transcriptional regulation. Moreover, non-hypusinated eIF5A was found to accumulate at the onset of apoptosis in animal cells (Jin et al., 2003). These findings suggest that the functional protein levels of eIF5A are thoroughly regulated implying that eIF5A must play an essential role in the irreversible initiation of programmed cell death. The pattern of eIF5A-2 protein expression observed during virulent infection in the present study could be described as climacteric, with slight up-regulation at 24 hours post-inoculation and more intense expression at 72 hours (Fig. 3). Perhaps the

initial wave of expression of eIF5A-2 is able to propagate an even stronger phase in expression by shuttling its own mRNA out of the nucleus. The stronger phase coincides temporally with a late stage of infection by *P. syringae*, which reportedly precedes the culminating host cell death phase (Xu and Roossinck, 2000). However, the climacteric pattern of expression was not observed in plants grown under the inherent long-day photoperiod (Fig.7), which showed a single strong up-regulation of eIF5A-2 at 72 hours.

The short-day light regime is favored over the inherent long-day regime in studying *Arabidopsis*-*P. syringae* interactions because it stimulates vegetative growth, resulting in more robust rosettes with multiple leaves. However, short-day light conditions have been linked to increased lignification and stress-like responses in *Arabidopsis* (Weretilnyk et al., 1995). Under stress, *Arabidopsis* can exhibit age-related resistance (ARR), where it becomes more resistant, or less susceptible, to virulent *Pseudomonas syringae* as it matures (Kus et al., 2002). Also, extensive lignification is part of the host defense response to pathogens and can interfere with bacterial growth within the apoplast (Newman et al., 2004). Increased lignification may result in a decrease in substomatal chamber volume as well as in the overall apoplast volume that accommodates the inoculum.

It has been proposed that *Pst* may undergo a short biotrophic phase within the apoplast, where it would feed on the small supply of nutrients present, before initiating its necrotrophic phase (personal communication from Dr. P. Goodwin, University of Guelph). This biotrophic phase may be shorter in duration in plants with increased lignification in which case the necrotrophic phase would commence earlier. Therefore, the more gradual increase in expression of eIF5A-2 in short-day plants may be attributed to the lower initial number of infiltrated bacteria and shorter biotrophic phase, leading to early initiation of host cell death to release nutrients. This theory is supported by the presence of lower bacterial population numbers within the short-day plant tissue. It is likely that the slower

population growth in addition to the stronger general resistance allowed for a rapid induction of the specific pathogen resistance response, halting the systemic spread of the pathogen.

The localization of eIF5A-2 in *Arabidopsis* leaves exposed to avirulent *Pst* is confined to the clusters of cells close to the vasculature as well as the bundle sheath cells (Fig.6A) even though the bacteria were infiltrated through stomates and filled the entire apoplast of the leaf. This is consistent with reports of preferential death of cells close to the vascular bundles following exposure to O₃, which can mimic HR in *Arabidopsis* (Wohlgemuth et al., 2002). It is proposed that the cells in the periveinal region might be disposed to amplified reactive oxygen species (ROS) production and thus, in the event of avirulent infection, undergo PCD to limit pathogen spread via the vascular system (Schraudner et al., 1998).

How cell death is induced during susceptible interactions is poorly understood. Recently, several studies indicate that the host may genetically condition pathogen susceptibility. For example, *Arabidopsis* POWDERY MILDEW RESISTANT (PMR) genes have been suggested to potentially encode host susceptibility factors. The *pmr* mutants are resistant to the normally virulent pathogen *Erysiphe cichoracearum*. These mutants neither develop lesions nor constitutively express elevated levels of PR1 or PDF1.2 (Vogel and Somerville, 2000). The *Arabidopsis* *dth9* mutant constitutively activates the promoter of CEVI-1, a gene from tomato that is induced only during compatible plant-virus interaction, suggesting that DETACHMENT 9 (DTH9) may function as a regulator of host disease susceptibility (Mayda et al., 2000a, Mayda et al., 2000b). The plant hormone ethylene has been implicated in the development of disease symptoms (Bent et al., 1992, Lund et al., 1998). Thus, the possible involvement of hormones and other host factors in modulating the cell death response to pathogens argues for a plant genetic program(s) that controls cell death during disease.

Screening of transgenic *Arabidopsis* plants exhibiting suppressed expression of eIF5A-2 following inoculation with the virulent strain of *Pseudomonas syringae* pv. *tomato* DC3000 helped uncover resistance phenotypes to the pathogen under conditions that normally result in necrotic disease (Fig. 14). Transgenic lines exhibited up to 99% decrease in bacterial population growth, relative to the wild type plants, which provides compelling evidence that eIF5A-2 plays an important role in regulating the response to the pathogen effector proteins delivered into the host cells upon infection.

A decline in chlorophyll levels within the infected tissue is one of the characteristic symptoms of disease. This chlorosis is believed to be caused by coronatine, a molecular mimic of JA and a virulence factor produced by strains of *P. syringae* (Bender et al., 1987, Feys et al., 1994, Hendrickson et al., 2000). A recent discovery of *Arabidopsis* CORONATINE-INSENSITIVE1 (COI1) gene through mutant analysis led to the proposal that a host-encoded component may play an active role during compatible interactions. The gene was shown to be required for disease symptom development following virulent *Pst* infection (Kloek et al., 2001). It has been proposed that the delivery of coronatine by pathogen into the plant cell may result in hyper-induction of the JA pathway and the concomitant suppression of SA-related defenses, which decreases plant fitness to such an extent that it cannot mount a proper defense to the pathogen (Felton et al., 1999a, Felton et al., 1999b, Pieterse and van Loon, 1999, Thomma et al., 2001). Accordingly, quantitative measurements of changes in chlorophyll levels as well as visual observations of chlorosis were used to assess the temporal progression of disease in wild type and transgenic plants in the present study. The levels of chlorophyll at 72 hours post inoculation for wild type plants were low, relative to the untreated control plants and plants of transgenic line 8A (Fig.15), indicating that *P. syringae* was not able to mount disease symptoms attributable to coronatine due to decreased population numbers. However,

visual observations have shown that the chlorosis symptoms were not fully eradicated in transgenic lines (Fig.16), leading to the conclusion that coronatine acts independently from eIF5A-2.

The extent and pattern of localization of eIF5A-2 expression in transgenic plants indicate that some translation still occurred following virulent infection, mainly around the stomate, which provided a point of entrance for the bacteria (Fig. 18B). The suppressed levels of eIF5A-2 prevented the *P. syringae* from inducing cell death in order to maintain growth, which in turn impeded the colonization of the pathogen beyond the substomatal space. It is plausible to assume that the substomatal space, being relatively more spacious than the rest of the apoplast, would contain more nutrients to accommodate the pathogen as it progressed through its biotrophic phase. This initial biotrophic phase is symptomless and lasts for about 24 hours post inoculation: however it does entail some pathogen growth. The cells adjoining the substomatal area were under severe stress from the effector proteins being delivered by the pathogen, which resulted in subsequent induction of eIF5A-2, although the levels were not detectable by Western blot analysis (Fig. 13).

In summary, the eIF5A-2 isoform was identified to be involved in signaling during pathogen challenge and wounding in *Arabidopsis*. The protein appears to be under post-transcriptional regulation and was shown to be strongly up-regulated during virulent infection with *Pseudomonas syringae* pv. *tomato* DC 3000 as well as through wounding. Transgenic plants with suppressed eIF5A-2 levels exhibited abrogation of disease development in response to a virulent infection demonstrated by reduced bacterial colonization and subsequent lack of chlorosis. Since *P. syringae* is a necrotroph, meaning that it requires the initiation of plant cell death to colonize in the plant, its inability to multiply in transgenic plants with suppressed levels of eIF5A-2 protein suggests that eIF5A-2 plays an important role in cell death induced by virulent infection. eIF5A-2 may be one of the links between the initial signals that elicit the transcription of cell death genes and their rapid and orchestrated translation.

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